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<b>(21) International Application Number:</b> PCT/US93/05629 <b>(22) International Filing Date:</b> 11 June 1993 (11.06.93)  <b>(30) Priority data:</b> 897,648                      12 June 1992 (12.06.92)      US 987,890                      8 December 1992 (08.12.92)    US 030,897                      15 March 1993 (15.03.93)      US  <b>(71) Applicant:</b> DNX CORPORATION [US/US]; 303B College Road East, Princeton Forrestal Area, Princeton, NJ 08540 (US).  <b>(72) Inventors:</b> LOGAN, John, S. ; 347 B Old York Road, Robbinsville, NJ 08691 (US). HOLTZMAN, Steven, H. ; 15 Sherbrooke Drive, Princeton Junction, NJ 08550 (US). O'DONNELL, J., Kevin ; 2822 Hearth Place, Doylestown, PA 18901 (US). PILDER, Stephen, H. ; 63-10 Raven's Crest Drive, Plainsboro, NJ 08536 (US). PINKERT, Carl, A. ; 1998 Lakemont Drive, Bessemer, AL 35023 (US). SWANSON, Mark, E. ; 16 Hereford Drive, Princeton Junction, NJ 08550 (US). KELLER, Hilary ; 3613 Quail Ridge Drive, Plainsboro, NJ 08536 (US). SHARMA, Ajay ; 24 Feiler Court, Lawrenceville, NJ 08648 (US). PARSONS, Cynthia, T. ; 26 Groendyke Lane, Plainsboro, NJ 08536 (US). KUMAR, Ramesh ; 60 Yard Road, Pennington, NJ 08534 (US). WHITE, Steven, P. ; 566 Edison Drive, Highstown, NJ 08520 (US).		<b>(74) Agents:</b> MISROCK, S., Leslie et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York; NY 10036 (US).  <b>(81) Designated States:</b> AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS  <b>(57) Abstract</b>  <p>The present invention relates to the use of transgenic pigs for the production of human hemoglobin in which, in certain embodiments, the pig beta globin promoter is used to facilitate the expression of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.</p>		

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PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

5 1. INTRODUCTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

10 2. BACKGROUND OF THE INVENTION

2.1. HEMOGLOBIN

15 Oxygen absorbed through the lungs is carried by hemoglobin in red blood cells for delivery to tissues throughout the body. At high oxygen tensions, such as those found in the proximity of the lungs, oxygen binds to hemoglobin, but is released in areas of low oxygen tension, where it is needed.

Each hemoglobin molecule consists of two alpha globin and two beta globin subunits. Each subunit, in turn, is noncovalently associated with an iron-containing heme group capable of carrying an oxygen molecule. Thus, each hemoglobin tetramer is capable of binding four molecules of oxygen. The subunits work together in switching between two conformational states to facilitate uptake and release of oxygen at the lungs and tissues, respectively. This effect is commonly referred to as heme-heme interaction or cooperativity.

20 The hemoglobins of many animals are able to interact with biologic effector molecules that can further enhance oxygen binding and release. This enhancement is manifested in changes which affect the allosteric equilibrium between the two conformational states of hemoglobin. For example, human and pig hemoglobin can bind 2,3-diphosphoglycerate (2,3 DPG),

which influences the equilibrium between the two conformational states of the tetramer and has the net effect of lowering the overall affinity for oxygen at the tissue level. As a result, 2,3-DPG increases the efficiency of oxygen delivery to the tissues.

## 2.2. GLOBIN GENE EXPRESSION

Hemoglobin protein is expressed in a tissue specific manner in red blood cells where it accounts for approximately ninety percent of total cellular protein. Thus, red blood cells, which have lost their nucleus and all but a minimal number of organelles, are effectively membrane-enclosed packets of hemoglobin dedicated to oxygen transfer.

Humans and various other species produce different types of hemoglobin during embryonic, fetal, and adult developmental periods. Therefore, the factors that influence globin gene expression must be able to achieve tissue specific control, quantitative control, and developmentally regulated control of globin expression.

Human globin genes are found in clusters on chromosome 16 for alpha ( $\alpha$ ) globin and chromosome 11 for beta ( $\beta$ ) globin. The human beta globin gene cluster consists of about 50 kb of DNA that includes one embryonic gene encoding epsilon ( $\epsilon$ ) globin, two fetal genes encoding gamma ( $\gamma$ ) G and gamma A globin, and two adult genes encoding delta ( $\delta$ ) and beta ( $\beta$ ) globin, in that order (Fritsch et al., 1980, Cell 19:959-972).

It has been found that DNA sequences both upstream and downstream of the  $\beta$  globin translation initiation site are involved in the regulation of  $\beta$  globin gene expression (Wright et al., 1984, Cell 38:263). In particular, a series of four Dnase I super hypersensitive sites (now referred to as the locus control region, or LCR) located about 50

kilobases upstream of the human beta globin gene are extremely important in eliciting properly regulated beta globin-locus expression (Tuan et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 83:1359-1363; PCT Patent Application WO 8901517 by Grosveld; Behringer et al., 1989, Science 245:971-973; Enver et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037; Hanscombe et al., 1989, Genes Dev. 3:1572-1581; Van Assendelft et al., 1989, Cell 56:967-977; Grosveld et al., 1987, Cell 51:975-985).

2.3.3. THE NEED FOR A BLOOD SUBSTITUTE

Recently, the molecular aspects of globin gene expression have met with even greater interest as researchers have attempted to use genetic engineering to produce a synthetic blood that would avoid the pitfalls of donor generated blood. In 1988, between 12 million and 14 million units of blood were used in the United States alone (Andrews, February 18, 1990, New York Times), an enormous volume precariously dependent on volunteer blood donations. About 5 percent of donated blood is infected by hepatitis virus (Id.) and, although screening procedures for HIV infection are generally effective, the prospect of contracting transfusion related A.I.D.S. remains a much feared possibility. Furthermore, transfused blood must be compatible with the blood type of the transfusion recipient; the donated blood supply may be unable to provide transfusions to individuals with rare blood types. In contrast, hemoglobin produced by genetic engineering would not require blood type matching, would be virus-free, and would be available in potentially unlimited amounts. Several research groups have explored the possibility of expressing hemoglobin in microorganisms. For example, see International Application No. PCT/US88/01534 by Hoffman and Nagai, which presents, in working

examples, production of human globin protein in E. coli.

5 2.4. TRANSGENIC ANIMALS

A transgenic animal is a non-human animal containing at least one foreign gene, called a transgene, in its genetic material. Preferably, the transgene is contained in the animal's germ line such that it can be transmitted to the animal's offspring. A number of techniques may be used to introduce the transgene into an animal's genetic material, including, but not limited to, microinjection of the transgene into pronuclei of fertilized eggs and/or manipulation of embryonic stem cells (U.S. Patent Nov 4,873,191 by Wagner and Hoppe; Palmiter and Brinster, 1986, Annu Rev. Genet. 20:465-499; French Patent Application 2593827 published August 7, 1987). Transgenic animals may carry the transgene in all of their cells or may be genetically mosaic.

Although the majority of studies have involved transgenic mice, other species of transgenic animal have also been produced, such as rabbits, sheep, pigs (Hammer et al., 1985, Nature 315:680-683) and chickens (Salter et al., 1987, Virology 157:236-240). Transgenic animals are currently being developed to serve as bioreactors for the production of useful pharmaceutical compounds (Van Brunt, 1988, Bio/Technology 6:1149-1154; Wilmut et al., 1988, New Scientist (July 7 issue) pp. 56-59).

Methods of expressing recombinant protein via transgenic livestock have an important theoretical advantage over protein production in recombinant bacteria and yeast; namely, the ability to produce large, complex proteins in which post-translational modifications, including glycosylation, phosphorylation, subunit assembly, etc. are critical for the activity of the molecule.

In practice, however, the creation of transgenic livestock has proved problematic. Not only is it technically difficult to produce transgenic embryos, but mature transgenic animals that produce significant quantities of recombinant protein may prove inviable. In pigs in particular, the experience has been that pigs carrying a growth hormone encoding transgene (the only transgene introduced into pigs prior to the present invention) suffered from a number of health problems, including severe arthritis, lack of coordination in their rear legs, susceptibility to stress, anoestrus in gilts and lack of libido in boars (Wilmut et al., supra). This is in contrast to transgenic mice carrying a growth hormone transgene, which appeared to be healthy (Palmiter et al., 1982, Nature 300:611-615). Thus, prior to the present invention, healthy transgenic pigs (which efficiently express their transgene(s)) had not been produced.

## 2.5. EXPRESSION OF GLOBIN GENES IN TRANSGENIC ANIMALS

Transgenic mice carrying human globin transgenes have been used in studying the molecular biology of globin gene expression. A hybrid mouse/human adult beta globin gene was described by Magram et al. in 1985 (Nature 315:338-340). Kollias et al. then reported regulated expression of human gamma-A, beta, and hybrid beta/gamma globin genes in transgenic mice (1986, Cell 46:89-94). Transgenic mice expressing human fetal gamma globin were studied by Enver et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037) and Constantoulakis et al. (1991, Blood 77:1326-1333). Autonomous developmental control of human embryonic globin gene switching in transgenic mice was observed by Raich et al. (1990, Science 250:1147-1149).

Transgenic mouse models for a variety of disorders of hemoglobin or hemoglobin expression have

been developed, including sickle cell disease (Rubin et al., 1988, Am. J. Human Genet. 42:585-591; Greaves et al., 1990, Nature 343:183-185; Ryan et al., 1990, Science 247:566-568; Rubin et al., 1991, J. Clin. Invest. 87:639-647); thalassemia (Anderson et al., 1985, Ann. New York Acad. Sci. (USA) 445:445-451; Sorenson et al., 1990, Blood 75:1333-1336); and hereditary persistence of fetal hemoglobin (Tanaka et al., 1990, Ann. New York Acad. Sci. (USA) 612:167-178).

Concurrent expression of human alpha and beta globin has led to the production of human hemoglobin in transgenic mice (Behringer et al., 1989, Science 245:971-973; Townes et al., 1989, Prog. Clin. Biol. Res. 316A:47-61; Hanscombe et al., 1989, Genes Dev. 3:1572-1581). It was observed by Hanscombe et al. (supra) that transgenic fetuses with high copy numbers of a transgene encoding alpha but not beta globin exhibited severe anemia and died prior to birth. Using a construct with both human alpha and beta globin genes under the control of the beta globin LCR, live mice with low copy numbers were obtained (Id.). Metabolic labeling experiments showed balanced mouse globin synthesis, but imbalanced human globin synthesis, with an alpha/beta biosynthetic ratio of about 0.6 (Id.).

### 3. SUMMARY OF THE INVENTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin and/or human globin. It is based, at least in part, on the discovery that transgenic pigs may be generated that express human hemoglobin in their erythrocytes and are healthy, suffering no deleterious effects as a result of heterologous hemoglobin production.

In particular embodiments, the present invention provides for transgenic pigs that express



human globin genes. Such animals may be used as a particularly efficient and economical source of human hemoglobin, in light of (i) the relatively short periods of gestation and sexual maturation in pigs; (ii) the size and frequency of litters, (iii) the relatively large size of the pig which provides proportionately large yields of hemoglobin; and (iv) functional similarities between pig and human hemoglobins in the regulation of oxygen binding and affinity which enables the transgenic pigs to remain healthy in the presence of high levels of human hemoglobin.

The present invention also provides for recombinant nucleic acid constructs that may be used to generate transgenic pigs. In preferred embodiments, such constructs place the human alpha and beta globin genes under the same promoter so as to avoid deleterious effects of globin chain imbalance and/or titration of transcription factors due to constitutive  $\beta$ -globin promoter activity in an inappropriate cell type (e.g. a primitive erythrocyte). In other preferred embodiments of the invention, the constructs comprise the pig adult beta globin gene regulatory region, comprising the promoter or the 3' region of the pig beta globin gene.

In an additional embodiment, the present invention provides for a hybrid hemoglobin that comprises human  $\alpha$  globin and pig  $\beta$  globin. The whole blood from transgenic pigs expressing this hybrid hemoglobin appears to exhibit a  $P_{50}$  that is advantageously higher than that of native human or pig blood.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as

to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells; and (iv) subjecting the released contents of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

#### 4. DESCRIPTION OF THE FIGURES

Figure 1. Recombinant nucleic acid constructs.

A. Construct  $\alpha\alpha\beta$  (the "116" construct); B. Construct  $\alpha\beta\beta$  (the "185" construct); C. Construct  $\beta\beta\alpha$  (the "290" construct); D. Construct  $\epsilon\beta\beta\alpha$ ; E. Construct  $\beta\epsilon\alpha\beta\beta$ ; F. Construct  $\alpha\beta\beta$  carrying a  $\beta 108$  Asn  $\rightarrow$  Asp mutation (the "hemoglobin Yoshizuka construct"); G. Construct  $\alpha\beta\beta$  carrying a  $\beta 108$  Asn  $\rightarrow$  Lys mutation (the "hemoglobin Presbyterian construct"); H. Construct  $\alpha\beta\beta(\Delta\alpha)$  coinjected with LCR  $\alpha$  (the "285" construct); I. Construct  $\alpha\beta\beta$  carrying an  $\alpha 134$  Thr  $\rightarrow$  Cys mutation (the "227" construct); J. Construct  $\alpha\beta\beta$  carrying an  $\alpha 104$  Cys  $\rightarrow$  Ser mutation (the "227" construct), a  $\beta 93$  Cys  $\rightarrow$  Ala mutation, and a  $\beta 112$  Cys  $\rightarrow$  Val mutation (the "228" construct); K. Construct  $\alpha\beta\delta$  (the "263" construct); and L. Construct  $\alpha\beta\delta(\Delta\alpha)$  coinjected with LCR  $\alpha$  (the "274" construct); M. Construct LCR  $\alpha$  coinjected with LCR  $\epsilon\beta$  (the "240" construct); N. Construct  $\alpha\beta\beta$  carrying a  $\beta 61$  Lys  $\rightarrow$  Met mutation (the "Hemoglobin Bologna" construct); O. Construct LCR  $\epsilon\alpha\beta$  (the "318" construct); P. Construct LCR  $\alpha\epsilon\beta$  (the "319" construct); Q. Construct LCR  $\alpha\alpha\epsilon\beta$  (the "329" construct); R. Construct LCR  $\alpha\epsilon(\beta\beta\beta)\beta$  (the

"339" construct); S. Construct  $\alpha\beta$  carrying an  $\alpha 75$  Asp  $\rightarrow$  Cys mutation (the "340" construct); T. Construct  $\alpha\beta$  carrying an  $\alpha 42$  Tyr  $\rightarrow$  Arg mutation (the "341" construct); U. Construct LCR  $\epsilon\beta\alpha$  (the "343" construct); V. Construct LCR  $\epsilon\beta\alpha$  (the "347" construct); W. Construct  $\alpha\beta$  carrying an  $\alpha 42$  Tyr  $\rightarrow$  Lys mutation; X. Construct  $\alpha\beta$  carrying an  $\alpha 42$  Tyr  $\rightarrow$  Arg mutation; and a  $\beta 99$  Asp  $\rightarrow$  Glu mutation; Y. Construct  $\alpha\beta$  carrying an  $\alpha 42$  Tyr  $\rightarrow$  Lys mutation; and a  $\beta 99$  Asp  $\rightarrow$  Glu mutation.

Figure 2. Transgenic pig.

Figure 3. Demonstration of human hemoglobin

expression in transgenic pigs. A. Isoelectric focusing gel analysis. B. Triton-acid urea gel of hemolysates of red blood cells representing human blood (lane 1); blood from transgenic pig 12-1 (lane 2), 9-3 (lane 3), and 6-3 (lane 4); and pig blood (lane 5) shows under-expression of human  $\beta$  globin relative to human  $\alpha$  globin in the transgenic animals.

Figure 4. Separation of human hemoglobin and pig hemoglobin by DEAE chromatography. A. Hemolyzed mixture of human and pig red blood cells; B. Hemolysate of red blood cells collected from transgenic pig 6-3. C. Human and mouse hemoglobin do not separate by DEAE chromatography under these conditions. D. Isoelectric focusing of human hemoglobin purified from pig hemoglobin.

Figure 5. Isoelectric focussing gel of reassociated pig hemoglobin (lane 1); reassociated pig/human hemoglobin mixture (lanes 2 and 4); reassociated human hemoglobin (lane 3); and transgenic pig hemoglobin (lane 5).

Figure 6. Separation of human hemoglobin by QCPH chromatography.

Figure 7. Oxygen affinity of transgenic hemoglobin.

- Figure 8. DNA sequence of the pig adult beta globin gene regulatory region, including the promoter region. Sequence extending to 869 base pairs upstream of the
- 5 ATG initiator codon (boxed) of the pig beta globin gene is shown. The position of the initiation of mRNA, the cap site, is indicated by an arrow. The sequences corresponding to GATA transcription factor binding sites are underlined.
- 10 Figure 9. Comparison of pig (top) and human (bottom) beta globin regulatory sequences. Differences in the two sequences are marked by asterisks.
- Figure 10. Graph depicting the percent homology between pig and human adult beta globin gene regulatory sequences, with base pair distance from the initiator codon mapped on the abscissa. A comparison of mouse and human sequences is also shown (dotted line with error bar).
- 15 Figure 11. Map of plasmid pgem5/Pig $\beta$ Pr(k) which contains the DNA sequence depicted in Figure 8.
- 20 Figure 12. Representation of the 339 and 354 cassettes for the production of human hemoglobin in transgenic pigs.
- Figure 13. Map of plasmid pSaf/Pig $\epsilon$ (k), containing the pig  $\epsilon$  gene.
- 25 Figure 14. Representation of the 426 and 427 expression cassettes for the production of  $\epsilon^{\text{pig}}$ ,  $\beta^{\text{human}}$  and  $\alpha^{\text{human}}$  hemoglobins in transgenic pigs.
- Figure 15. Iso-electric focussing gel of hemoglobin produced by transgenic pig 70-3, which carries the 339 construct, and by transgenic pig 6-3, which carries the 116 construct. Human hemoglobin is run as a standard.
- 30 Figure 16. Map of plasmid pig3' $\beta$  containing the 3' end of the pig beta globin gene.
- 35 Figure 17. Transgenic pigs obtained from construct "339" (See Figure 1R). Levels of human hemoglobin expression and copy number are shown.

Figure 18. Isoelectric focussing gel of hemoglobin levels in transgenic pigs obtained using construct "339".

5 Figure 19. Isoelectric focussing gel demonstrating levels of hemoglobin expression in representative transgene positive 38-4 offspring carrying the "185" construct (or  $\alpha\beta$  construct; see Figure 1B).

10 Figure 20. Molecular modeling of hybrid human  $\alpha$ /pig  $\beta$  and human  $\alpha$ /human  $\beta$  hemoglobin molecules.  $\beta$  subunits are in blue,  $\alpha$  subunits in red. Above the middle helix of the  $\beta$  human (blue) one can see a gap in the green contour (see arrow). In  
15 the hybrid this gap is filled in. This difference is due to a change at  $\beta 112$  Cys  $\rightarrow$  Val, where Valine contributes to greater hydrophobic interactions.

Figure 21. Molecular modeling demonstrating the differences at the  $\alpha\beta$  interface between a  $\beta$  globin containing Cys at position 112 (the yellow molecule) and a  $\beta$  globin with Val at position 112 (the white molecule). Cys is yellow, Val is white and the opposing  $\alpha$  interface is red. Val  
20 is flexible. One arm of its branch can easily move for a nearly perfect fit against the  $\alpha$  subunit residues. The yellow Cys is slightly further allowing for a small gap (see arrow). Biosyn's standard default Van der Waal's distance  
25 was used.

Figure 22. Purification of Hb Presbyterian from transgenic pig hemosylate.

Figure 23. Characterization of purified Hb Presbyterian by HPLC showing separation of the heme moiety, pig  $\alpha$  globin ("p alpha"), human beta globin ("h beta"), human alpha globin ("h alpha") and pig beta globin ("p beta").

Figure 24. Oxygen binding curve for Hb Presbyterian.

Figure 25: Purification of Hb-Yoshizuka from transgenic pig hemolysate.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a method of producing human hemoglobin that utilizes transgenic pigs, novel globin encoding nucleic acid constructs, and transgenic pigs that express human hemoglobin.

For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) preparation of globin gene constructs;

(ii) preparation of transgenic pigs;

(iii) preparation of human hemoglobin and its separation from pig hemoglobin; and

(iv) preparation of human/pig hybrid hemoglobin.

The invention also includes the preparation of

the invention also includes the preparation of

### 5.1. PREPARATION OF GLOBIN GENE CONSTRUCTS

The present invention provides for a method of producing human globin and/or hemoglobin in transgenic pigs. Human hemoglobin is defined herein

to refer to hemoglobin formed by globin chains encoded by human globin genes (including alpha, beta, delta, gamma, epsilon and zeta genes) or variants thereof

which are naturally occurring or the products of genetic engineering. Such variants are at least about

ninety percent homologous in amino acid sequence to a naturally occurring human hemoglobin. In preferred

embodiments, the human hemoglobin of the invention comprises a human alpha globin and a human beta globin chain. The human hemoglobin of the invention

comprises at least two different globin chains, but may comprise more than two chains, to form, for example, a tetrameric molecule, octameric molecule, etc. In preferred embodiments of the invention, human

hemoglobin consists of two human alpha globin chains and two human beta-globin chains. As discussed infra, the present invention also provides for hybrid hemoglobins comprising human  $\alpha$  globin and pig  $\beta$  globin. According to particular embodiments of the present invention, at least one human globin gene, such as a human alpha and/or a human beta globin gene, under the control of a suitable promoter or promoters, is inserted into the genetic material of a pig so as to create a transgenic pig that carries human globin in at least some of its red blood cells. This requires the preparation of appropriate recombinant nucleic acid sequences. In preferred embodiments of the invention, both human  $\alpha$  and human  $\beta$  genes are expressed. In an alternative embodiment, only human  $\alpha$  globin or human  $\beta$  globin is expressed. In further embodiments, human embryonic or fetal globin genes are expressed or are used as developmental expression regulators of adult genes.

Human alpha and beta globin genes may be obtained from publicly available clones, e.g. as described in Swanson et al., 1992, Bio/Technol. 10:557-559. Nucleic acid sequences encoding human alpha and beta globin proteins may be introduced into an animal via two different species of recombinant constructs, one which encodes human alpha globin, the other encoding human beta globin; alternatively, and preferably, both alpha and beta-encoding sequences may be comprised in the same recombinant construct. The pig epsilon globin gene is contained in plasmid p $\epsilon$ sa $\epsilon$ /pig  $\epsilon$  (k) (Figure 13), deposited with the ATCC and assigned accession number 75373.

A suitable promoter, according to the invention, is a promoter which can direct transcription of human alpha and/or beta globin genes in red blood cells. Such a promoter is preferably

selectively active in erythroid cells. This would include, but is not limited to, a globin gene promoter, such as the human alpha, beta, delta, epsilon or zeta promoters, or a globin promoter from another species. It may, for example, be useful to utilize pig globin promoter sequences. For example, as discussed in Section 10, *infra*, the use of the endogenous pig  $\beta$  globin gene control region, as contained in plasmid Pgem5/Pig $\beta$ pr(K), deposited with the ATCC and assigned accession number 75371 and having the sequence set forth in Figure 8, has been shown to operate particularly efficiently. The human alpha and beta globin genes may be placed under the control of different promoters, but, since it has been inferred that vastly different levels of globin chain production may result in lethality, it may be preferable to place the human alpha and beta globin genes under the control of the same promoter sequence. In order to avoid chain imbalance and/or titration of transcription factors due to constitutive  $\beta$ -globin promoter activity in an inappropriate cell type, it is desirable to design a construct which leads to coordinate expression of human alpha and beta globin genes at the same time in development and at quantitatively similar levels.

In one particular, non-limiting embodiment of the invention, a construct comprising the  $\alpha\alpha\beta$  construct (also termed the "116" construct; Swanson et al., 1992, Bio/Technol. 10:557-559; see Figure 1A) may be utilized. Although this construct, when present as a transgene at high copy number, has resulted in deleterious effects in mice, it has been used to produce healthy transgenic pigs (see Example Section 6, *infra*).

In another particular, non-limiting embodiment of the invention, a construct comprising the  $\alpha\beta$  sequence (also termed the "185" construct; see



Figure 1B) may be used. Such a construct has the advantage of placing both alpha and beta globin-encoding sequences under the control of the same promoter (the alpha globin promoter).

In another particular, non-limiting embodiment of the invention, a construct coding for di-alpha globin-like polypeptides may be introduced to form transgenic pigs that produce human hemoglobins with decreased dimerization and an increased half-life (WO Patent 9013645).

In yet another particular, non-limiting embodiment of the invention, a construct comprising the human adult alpha globin and epsilon globin gene, the pig beta globin gene control region and the human beta globin gene (the "339" construct, see Figure 1R) may be used.

Furthermore, the incorporation of a human or pig epsilon globin gene into the construct may facilitate the production of high hemoglobin levels. The pig epsilon globin gene may permit correct developmental regulation of the adult  $\beta$  globin gene. High levels of expression of introduced adult alpha globin gene(s) may result in a chain imbalance problem during intrauterine development of a transgenic pig embryo (because an adult beta globin gene in the construct would not yet be expressed) thereby compromising the viability of the embryo. By providing high levels of embryonic globins during development, the viability of such embryos may be improved. The pig epsilon globin gene, as contained in plasmid pSaf/Pige, deposited with the ATCC and assigned accession number 75373, is shown in Figure 13.

The present invention, in further specific embodiments, provides for (i) the construct  $\beta\alpha$ , in which the human alpha and beta globin genes are driven by separate copies of the human beta globin promoter

(Figure 1C); (ii) the  $\epsilon\beta\alpha$  construct, which comprises human embryonic genes zeta and epsilon under the control of the epsilon promoter and both alpha and beta genes under the control of the beta promoter; (Figure 1D); (iii) the  $\beta\epsilon\alpha\beta$  construct, which comprises human embryonic genes zeta and epsilon under the control of the zeta promoter and both alpha and beta genes under the control of the alpha promoter; (Figure 1E); (iv) the  $\alpha\beta$  construct carrying a mutation that results in an aspartic acid residue (rather than an asparagine residue) at amino acid number 108 of  $\beta$ -globin protein, to produce hemoglobin Yoshizuka (Figure 1F, construct "294"); (v) the  $\alpha\beta$  construct carrying a mutation that results in a lysine residue (rather than an asparagine residue) at amino acid number 108 of  $\beta$ -globin protein, to produce hemoglobin Presbyterian (Figure 1G, construct "293"); (vi) the  $\alpha\beta(\Delta\alpha)$  construct, coinjected with LCR  $\alpha$ , which comprises the human  $\beta$ -globin gene under the control of the human  $\alpha$ -globin promoter and a separate nucleic acid fragment comprising the human  $\alpha$ -globin gene under its own promoter (Figure 1H); (vii) the  $\alpha\beta$  construct carrying a mutation that results in a cysteine residue (rather than a threonine residue) at amino acid number 134 of  $\alpha$ -globin protein (Figure 1I); (viii) the  $\alpha\beta$  construct carrying a mutation that results in a serine residue (rather than a cysteine residue) at amino acid number 104 of the  $\alpha$ -globin protein, an alanine residue (rather than a cysteine residue) at amino acid number 93 of the  $\beta$ -globin protein and a valine residue (rather than a cysteine residue) at amino acid number 112 of the  $\beta$ -globin protein (Figure 1J); (ix) the  $\alpha\delta$  construct, which comprises the human adult  $\alpha$ -globin promoter under its own promoter and the human  $\delta$ -globin gene under the control of the human adult  $\alpha$ -globin promoter (Figure 1K); (x) Construct  $\alpha\delta(\Delta\alpha)$  coinjected with LCR  $\alpha$ ,

which comprises the human  $\delta$ -globin gene under the control of the human  $\alpha$ -globin promoter and a separate nucleic acid fragment comprising the human  $\alpha$ -globin gene under its own promoter (Fig. 1L); (xi) Construct LCR  $\alpha$  coinjected with LCR  $\epsilon\beta$ , which comprises the human  $\alpha$ -globin gene under the control of its own promoter and a separate nucleic acid fragment comprising the human embryonic  $\epsilon$ -globin gene and the adult  $\beta$ -globin gene under the control of their own promoters (Fig. 1M); (xii) the  $\alpha\beta$  construct carrying a mutation that results in a methionine residue (rather than a lysine residue) at amino acid number 61 of the  $\alpha$ -globin protein (Fig. 1N); (xiii) the  $\epsilon\alpha\beta$  construct, which comprises the human embryonic epsilon gene, the human adult alpha globin gene and the human adult beta globin gene linked in tandem from 5' to 3' (Fig. 1O); (xiv) the  $\alpha\epsilon\beta$  construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5' to 3' (Fig. 1P); (xv) the  $\alpha\alpha\epsilon\beta$  construct, which comprises two copies of the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5' to 3' (Fig. 1Q); (xvi) the  $\alpha\epsilon(\beta\beta)\beta$  construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene under the control of the endogenous porcine adult beta globin promoter all linked in tandem from 5' to 3' (Fig. 1R); (xvii) the  $\alpha\beta$  construct carrying a mutation that results in a cysteine residue (rather than an aspartic acid residue) at amino acid number 75 of the  $\alpha$ -globin protein (Fig. 1S); (xviii) the  $\alpha\beta$  construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the  $\alpha$ -globin protein (Fig. 1T); (xvix) the LCR  $\epsilon\beta\alpha\alpha$  construct, which comprises

the human embryonic epsilon-globin gene, the human adult beta globin gene and two copies of the human adult alpha-globin gene linked in tandem from 5' to 3' (Fig. 1U); (xx) the LCR  $\epsilon\beta\alpha$  construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and the human adult alpha-globin gene linked in tandem from 5' to 3' (Fig. 1V); (xxi) the  $\alpha\beta$  construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the  $\alpha$ -globin protein (Fig. 1W); (xxii) the  $\alpha\beta$  construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the  $\alpha$ -globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the  $\beta$ -globin protein (Fig. 1X); (xxiii) the  $\alpha\beta$  construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the  $\alpha$ -globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the  $\beta$ -globin protein (Fig. 1Y); and (xxiv) the  $\alpha^{\text{pig}}\epsilon(\text{pig}\beta\text{p})\beta$  construct comprising the pig epsilon-globin gene and beta globin control region (constructs 426 and 427, Figure 14).

In transgenic pigs expressing human hemoglobin three types of hemoglobin dimers are detectable: pig  $\alpha$ /pig  $\beta$ , human  $\alpha$ /human  $\beta$ , and hybrid human  $\alpha$ /pig  $\beta$ . In certain embodiments of the invention, it may be desirable to decrease the amount of hybrid hemoglobin. Accordingly, the molecular basis for the formation of hybrid hemoglobin has been investigated using molecular modeling studies. Based on the information derived from these studies, the human alpha and beta globin structures can be modified to increase the level of human  $\alpha$ /human  $\beta$  dimers (See Section 11.), so that in further embodiments of the

invention, constructs comprising the  $\alpha\beta$  sequence may be modified to code for  $\alpha$  or  $\beta$  globin proteins carrying amino acid changes that will lead to increases in the level of human  $\alpha$ /human  $\beta$  hemoglobin dimers in transgenic pigs. The present invention, provides for constructs which encode human  $\alpha$  globin and human  $\beta$  globin carrying one or more of the following mutations in the  $\alpha$  globin molecule: (1) a Thr at position 30 instead of Glu; (ii) a Tyr at position 36 instead of Phe; (iii) a Phe instead of Leu at position 106; (iv) a Ser or Cys instead of Val at position 107; and/or (v) a Cys instead of Ala at position 111. In specific embodiments, the construct carrying such mutation(s) is the  $\alpha\beta$  construct. The present invention, in further embodiments, provides for constructs which encode human  $\alpha$  globin and human  $\beta$  globin carrying one or more of the following mutations in the  $\beta$  globin molecule: (1) a Leu instead of Val at position 33; (ii) a Val or Ile instead of Cys at position 112; (iii) a Val or Leu instead of Ala at position 115; (iv) a His instead of Gly at position 119; (v) a Met instead of Pro at position 125; (vi) an Ile instead of Ala at position 128; and/or (vii) a Glu instead of Gln at position 131; and/or (viii) a Glu instead of Gln at position 131. In specific embodiments, the construct carrying the mutation(s) is the  $\alpha\beta$  construct.

In further embodiments it may be desirable to include, in constructs, the untranslated 3' end of the pig beta globin gene as contained in plasmid pPig3 $\beta$  (Figure 16) as deposited with the ATCC and assigned accession number 75372. (see, for example, construct 354 in Figure 12 and Figures 426 and 427 in Figure 14). Such constructs may also be useful in the expression of non-globin protein in pig erythrocytes.

In further embodiments, the pig beta globin control region depicted in Figures 8 and 9 may be used

in constructs that encode non-globin proteins for the expression of said proteins in transgenic pig or other non-human erythrocytes.

5           The recombinant nucleic acid constructs described above may be inserted into any suitable plasmid, bacteriophage, or viral vector for amplification, and may thereby be propagated using methods known in the art, such as those described in  
10   Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. In the working examples presented below, the pUC vector (Vanish-Perron et al., 1985, Gene 103-119) was utilized. The present invention further provides for  
15   isolated and purified nucleic acids comprising the pig adult beta globin promoter regulatory region, the pig 3' beta globin region, and the pig epsilon globin gene as comprised, respectively, in plasmids pgem5/Pig $\beta$ pr(K) (ATCC accession no. 75371), pPig3' $\beta$  (ATCC accession no. 75372), and pSaf/pig $\epsilon$ (K) (ATCC accession no. 75373), respectively.

20           Constructs may desirably be linearized for preparation of transgenic pigs. Vector sequence may desirably be removed.

## 25           5.2. PREPARATION OF TRANSGENIC PIGS

          The recombinant constructs described above may be used to produce a transgenic pig by any method known in the art, including but not limited to, microinjection, embryonic stem (ES) cell manipulation,  
30   electroporation, cell gun, transfection, transduction, retroviral infection, etc. Species of constructs may be introduced individually or in groups of two or more types of construct.

35           According to a preferred specific embodiment of the invention, a transgenic pig may be produced by the methods as set forth in Example Section 6, infra. Briefly, estrus may be synchronized in sexually mature

gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all  
5 gilts may be given an intramuscular injection (IM) of prostaglandin F<sub>2α</sub> (Lutalyse: 10 mg/injection) at 0800 and 1600 hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single IM injection of pregnant mare serum  
10 gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) may be administered to all donors at 80 hours after PMSG. Following AT withdrawal, donor and recipient gilts may be checked twice daily for signs of estrus  
15 using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.  
20 Between 59 and 66 hours after the administration of HCG one- and two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of  
25 bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of  
30 the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova may be flushed in retrograde fashion by inserting a 20-g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered  
35 saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into sterile 17 x 100 mm polystyrene tubes.

Flushings may be transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova may be washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50  $\mu$ l drops of BMOC-3 medium under oil. Ova may be stored at 38°C under a 90% N<sub>2</sub>, 5% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere until microinjection is performed.

One- and two-cell ova may be placed in a Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5 - 10  $\mu$ l drop of HEPES medium under oil on a depression slide. Microinjection may be performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (linearized at a concentration of about 1ng/ $\mu$ l of Tris-EDTA buffer) may be injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova may be returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N<sub>2</sub>, 5% CO<sub>2</sub>, 5% O<sub>2</sub> atmosphere prior to their transfer to suitable recipients. Ova may preferably be transferred within 10 hours of recovery.

Only recipients which exhibit estrus on the same day or 24 hours later than the donors may preferably be utilized for embryo transfer. Recipients may be anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one-and/or two-cell ova and 4-6 control ova may be transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set may be connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium may be aspirated into the tubing. The tubing may then be fed through the ostium of the



oviduct until the tip reaches the lower third or isthmus of the oviduct. The ova may be subsequently expelled as the tubing is slowly withdrawn.

- 5 The exposed portion of the reproductive tract may be bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin may be sutured as three separate layers.
- 10 An uninterrupted Halstead stitch may be used to close the linea alba. The fat and skin may be closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (e.g. Furazolidone) may then be administered to the incision area.
- 15 Recipients may be penned in groups of about four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients may be checked daily for signs of estrus using a mature boar.
- 20 On day 35, pregnancy detection may be performed using ultrasound. On day 107 of gestation recipients may be transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing may be induced by the administration of prostaglandin  $F_2$  (10 mg/injection) at 0800 and 1400 hours on day 112 of
- 25 gestation. In all cases, recipients may be expected to farrow within 34 hours following  $PGF_2$  administration.

- Twenty-four hours after birth, all piglets
- 30 may be processed, i.e. ears notched, needle teeth clipped, 1 cc of iron dextran administered, etc. A tail biopsy and blood may also be obtained from each pig.

- Pigs produced according to this method are
- 35 described in Example Section 6, infra, and are depicted in Figure 2. Such pigs are healthy, do not appear to be anemic, and appear to grow at a rate comparable to that of their non-transgenic

littermates. Such pigs may transmit the transgene to their offspring.

Pigs having certain characteristics may be especially useful for the production of human hemoglobin; such pigs, examples of which follow, represent preferred, non-limiting, specific embodiments of the invention.

According to one preferred specific embodiment of the invention, a transgenic pig contains at least twenty copies of a globin transgene.

According to a second preferred specific embodiment, the  $P_{50}$  of whole blood of a transgenic pig according to the invention is increased by at least ten percent over the  $P_{50}$  of the whole blood of a comparable non-transgenic pig, taking into consideration factors such as altitude, oxygen concentrations, pregnancy, the presence of mutant hemoglobin, etc. Thus, the present invention provides for a non-pregnant transgenic pig that carries and expresses a human globin transgene in which the  $P_{50}$  of whole blood of the transgenic pig is at least ten percent greater than the  $P_{50}$  of whole blood of a comparable non-pregnant non-transgenic pig at the same altitude.

In other preferred specific embodiments, the present invention provides for a transgenic pig in which the amount of human globin produced relative to total hemoglobin is at least two percent, more preferably at least five percent, and most preferably at least ten percent.

Section 6, *infra*, describes transgenic pigs which serve as working examples of preferred, non-limiting, specific examples of the invention.

### 5.3. PREPARATION OF HUMAN HEMOGLOBIN AND ITS SEPARATION FROM PIG HEMOGLOBIN

The present invention provides for a method for producing human hemoglobin comprising introducing a transgene or transgenes encoding human hemoglobin, such as a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its blood cells.

10 The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human  
15 hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells to form a lysate; (iv) subjecting the  
20 lysate of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin; and (v) collecting the fractions that contain purified human hemoglobin. Such fractions may be identified by isoelectric  
25 focusing in parallel with appropriate standards. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

In order to prepare human hemoglobin from  
30 the transgenic pigs described above, red blood cells are obtained from the pig using any method known in the art. The red blood cells are then lysed using any method, including hemolysis in a hypotonic solution such as distilled water, or using techniques as  
35 described in 1981, Methods in Enzymology Vol. 76, and/or tangential flow filtration.

For purposes of ascertaining whether human hemoglobin is being produced by a particular

transgenic pig, it may be useful to perform a small-scale electrophoretic analysis of the hemolysate, such as, for example, isoelectric focusing using standard techniques. Alternatively, or for larger scale purification, human hemoglobin may be separated from pig hemoglobin using ion exchange chromatography. Surprisingly, as discussed in Section 7, supra, human hemoglobin was observed to readily separate from pig hemoglobin using ion exchange chromatography whereas mouse hemoglobin and human hemoglobin were not separable by such methods. Any ion exchange resin known in the art or to be developed may be utilized, including, but not limited to, resins comprising diethylaminoethyl, Q-Sepharose, QCPI (I.B.F.), Zephyr, Spherox, ectiola, carboxymethylcellulose, etc., provided that the resin results in a separation of human and pig hemoglobin comparable to that achieved using DEAE resin.

According to a specific, nonlimiting embodiment of the invention, in order to separate human from pig hemoglobin (including human/pig hemoglobin hybrids) to produce substantially pure human hemoglobin, a hemolysate of transgenic pig red blood cells, prepared as above may be applied to a DEAE anion exchange column equilibrated with 0.2 M glycine buffer at Ph 7.8 and washed with 0.2 M glycine Ph 7.8/5 Mm NaCl, and may then be eluted with a 5-30 Mm NaCl gradient, or its equivalent (see, for example, Section 9 infra). Surprisingly, despite about 85 percent homology between human and pig globin chains, human and pig hemoglobin separates readily upon such treatment, with human hemoglobin eluting earlier than pig hemoglobin. Elution may be monitored by optical density at 405 nm and/or electrophoresis of aliquots taken from serial fractions. Pig hemoglobin, as well as tetrameric hemoglobin composed of heterodimers

formed between pig and human globin chains, may be separated from human hemoglobin by this method. Human hemoglobin produced in a transgenic pig and separated from pig hemoglobin by this method has an oxygen binding capability similar to that of native human hemoglobin.

According to another specific, non-limiting embodiment of the invention, human hemoglobin may be separated from pig hemoglobin (including human/pig hemoglobin hybrids) using QCP1 ion exchange resin as follows:

About 10 mg of hemoglobin prepared from transgenic pig erythrocytes may be diluted in 20ml of Buffer A (Buffer A = 10mM Tris, 20mM Glycine Ph 7.5). This 20ml sample may then be loaded at a flow rate of about 5ml/min onto a QCP1 column (10ml) which has been equilibrated with Buffer A. The column may then be washed with 2 volumes of Buffer A, and then with 20 column volumes of a 0-50mM NaCl gradient (10 column volumes of Buffer A + 10 column volumes of 10mM Tris, 20mM Glycine, 50mM NaCl Ph 7.5) or, alternatively, 6 column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl, pH 7.5, and the O.D.<sub>280</sub> absorbing material may be collected in fractions to yield the separated hemoglobin, human hemoglobin being identified, for example, by isoelectric focusing using appropriate standards. The QCP1 column may be cleaned by elution with 2 column volumes of 10mM Tris, 20mM Glycine, 1M NaCl, pH 7.5.

For certain mutant hemoglobins, it may be desirable to utilize a modified purification procedure. Accordingly, for the separation of Hb Presbyterian from pig Hb, a procedure as described in Example Section 12.1, *infra*, may be used, and for separation of Hb Yoshizuka, a procedure as described in Example Section 12.2, *infra*, may be used.

#### 5.4. PREPARATION OF HUMAN/PIG HYBRID HEMOGLOBIN

The present invention also provides for essentially purified and isolated human/pig hybrid hemoglobin, in particular human  $\alpha$ /pig  $\beta$  hybrid hemoglobin. Pig  $\alpha$ /human  $\beta$  hybrid has not been observed to form either in vitro in reassociation experiments or in vivo in transgenic pigs.

The present invention provides for hybrid hemoglobin and its use as a blood substitute, and for a pharmaceutical composition comprising the essentially purified and isolated human/pig hemoglobin hybrid in a suitable pharmacological carrier.

Hybrid hemoglobin may be prepared from transgenic pigs, as described herein, and then purified by chromatography, immunoprecipitation, or any other method known to the skilled artisan. The use of isoelectric focusing to separate out hemoglobin hybrid is shown in Figures 3 and 5.

Alternatively, hybrid hemoglobin may be prepared using nucleic acid constructs that comprise both human and pig globin sequences which may then be expressed in any suitable microorganism, cell, or transgenic animal. For example, a nucleic acid construct that comprises the human  $\alpha$  and pig  $\beta$  globin genes under the control of a suitable promoter may be expressed to result in hybrid hemoglobin. As a specific example, human  $\alpha$  globin and pig  $\beta$  globin genes, under the control of cytomegalovirus promoter, may be transfected into a mammalian cell such as a COS cell, and hybrid hemoglobin may be harvested from such cells. Alternatively, such constructs may be expressed in yeast or bacteria.

It may be desirable to modify the hemoglobin hybrid so as to render it non-immunogenic, for example, by linkage with polyethylene glycol or by encapsulating the hemoglobin in a membrane, e.g. in a liposome.

6. EXAMPLE: GENERATION OF TRANSGENIC PIGS  
THAT PRODUCE HUMAN HEMOGLOBIN

6.1. MATERIALS AND METHODS

6.1.1. NUCLEIC ACID CONSTRUCTS

5. Constructs 116 (the  $\alpha\alpha\beta$  construct), 185 (the  $\alpha\beta\beta$  construct), 263 (the  $\alpha\beta\delta$  construct) 339, 293 and 294 were microinjected into pig ova as set forth below in order to produce transgenic pigs.

10. 6.1.2. PRODUCTION OF TRANSGENIC PIGS

15. Estrus was synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all  
20. gilts received an intramuscular injection (IM) of prostaglandin  $F_{2\alpha}$  (Lutalyse: 10 mg/injection) at 0800 and 1600. Twenty-four hours after the last day of AT consumption all donor gilts received a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) was administered to all donors at 80 hours after PMSG.

25. Following AT withdrawal, donor and recipient gilts were checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration were bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

30. Between 59 and 66 hours after the administration of HCG, one- and two-cell ova were surgically recovered from bred donors using the following procedure. General anesthesia was induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the  
35. reproductive tract was exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) was inserted into the ostium of the

oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova were flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) was infused into the oviduct and flushed toward the glass cannula. The medium was collected into sterile 17 x 100 mm polystyrene tubes. Flushings were transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild-M3 stereomicroscope. All one- and two-cell ova were washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50  $\mu$ l drops of BMOC-3 medium under oil. Ova were stored at 38°C under a 90% N<sub>2</sub>, 5% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere until microinjection was performed.

One- and two-cell ova were placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium-supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova were then transferred to a 5 -10  $\mu$ l drop of HEPES medium under oil on a depression slide. Microinjection was performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (1ng/ $\mu$ l of Tris-EDTA buffer) were injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova were returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N<sub>2</sub>, 5% CO<sub>2</sub>, 5% O<sub>2</sub> atmosphere prior to their transfer to suitable recipients. Ova were transferred within 10 hours of recovery.

Only recipients which exhibited estrus on the same day or 24 hours later than the donors were utilized for embryo transfer. Recipients were



anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one- and/or two-cell ova and 4-6 control ova were transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set was connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium were aspirated into the tubing. The tubing was then fed through the ostium of the oviduct until the tip reached the lower third or isthmus of the oviduct. The ova were subsequently expelled as the tubing was slowly withdrawn.

The exposed portion of the reproductive tract was bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin were sutured as three separate layers. An uninterrupted Halstead stitch was used to close the linea alba. The fat and skin were closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (Furazolidone) was then administered to the incision area.

Recipients were penned in groups of four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients were checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection was performed using ultrasound. On day 107 of gestation recipients were transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing was induced by the administration of prostaglandin  $F_{2\alpha}$  (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients farrowed within 34 hours following PGF<sub>2a</sub> administration.

Twenty-four hours after birth, all piglets were processed, i.e. ears were notched, needle teeth clipped, 1 cc of iron dextran was administered, etc.

A tail biopsy and blood were also obtained from each pig.

5 6.2. RESULTS AND DISCUSSION

10 of 3566 injected ova, thirteen transgenic pigs that expressed human hemoglobin were born, two of which died shortly after birth due to normal breeding-related incidents completely unrelated to the fact that they were transgenic pigs (Table I). The remaining 11 appeared to be healthy. A photograph of one transgenic pig is presented in Figure 2. Profiles of the pigs and of the percent "authentic" and "hybrid" human hemoglobin ("HB") produced are set  
15 forth in Table II, infra. Total hemoglobin was calculated as the sum of human  $\alpha\beta$  plus one-half of the human  $\alpha$  pig  $\beta$  hybrid. Figure 3 presents the results of isoelectric focussing and triton acid urea gels of hemoglobin produced by three of these pigs (numbers  
20 12-1, 9-3, and 6-3) which demonstrate the expression of human alpha and beta globin in these animals.

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30

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TABLE I

Efficiency of Transgenic Pig Production  
Human Hemoglobin Gene Construct(s)

Parameter	Total After 22 Trials
Total Ova Collected	8276
10 Total # Fertilized	7156
Total # Injected	3566
# Injected Ova Transferred	3566
# Control Ova Transferred	279
# Recipients Used	104
15 # Pigs Born (Male, Female)	208,332
# Transgenic (Male, Female)	8,5 (0.36) <sup>a</sup>
# Expressing	13

20 <sup>a</sup> Proportion of injected ova which developed into  
transgenic pigs (13 transgenics/3566 injected ova).

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30

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TABLE II  
FOUNDERS

5	PIG	GENDER	TRANSGENE CONSTRUCT	AUTHENTIC HUMAN HB	HYBRID HB	TOTAL HUMAN HB	COPY #
	6-3	F	116	6.2%	8.1%	10.3%	57
	9-3	F	116	1.0%	33.1%	16.6%	1
10	22-2	M	185	<1%	5.0%	5.0%	55
	33-7	F	185	*died shortly after birth			0.5
	38-1	F	185	1.0%	8.3%	5.2%	17
	38-3	M	185	4.7%	17.2%	13.2%	22
15	38-4	M	185	3.2%	7.0%	6.7%	5
	47-3	M	263	<1%	2.9%	2.0%	4-6
	47-4	F	263	<1%	18.5%	10.0%	1-2
	52-3	M	263	<1%	7.6%	4.0%	
	52-7	M	263	<1%	26.4%	13.0%	
20	53-11	M	263	<1%	15.5%	8.0%	
	70-3	F	339	23	31	38	3

25

30

35

Table III presents the profiles of offspring of pig number 9-3, which shows that the F1 generation of transgenic pigs are capable of expressing hemoglobin. Of note, none of the offspring of pig number 6-3 were found to be transgenic, possibly due to the absence of transgene in the animal's reproductive tissue.

Table IV presents hemoglobin expression data of offspring of pig 38-4 carrying the "185" construct (the " $\alpha\beta$ " construct; see Figure 1B). Table V presents a summary of the profiles of offspring of pig number 38-4 in which a large percentage (37.1%) of offspring were positive for expression of human hemoglobin indicating germ line transmission of the transgene. Figure 19 presents the results of isoelectric focussing which demonstrates the levels of hemoglobin expression in representative transgene positive 38-4 offspring.

- 36 -

TABLE III

F1 (OFFSPRING) OF FIG 9-3

PIG	GENDER	CONST.	AUTHENTIC HUMAN HB	HYBRID HUMAN HB	TOTAL HUM.	COPY #
9-3-1	F	116	1.0%	31.5%	16.0%	1
9-3-2*	F	116	1.0%	32.9%	17.0%	1
9-3-3	M	116	1.0%	29.7%	15.0%	1
9-3-4	M	116	1.0%	32.8%	17.0%	1
9-3-6	F	116	1.0%	29.1%	15.0%	1
9-3-8	M	116	1.0%	31.6%	16.0%	1
9-3-9	M	116	1.0%	30.2%	16.0%	1

15 \*9-3-2 died the day after birth.

100% 40

EXPRESSION LEVEL

39

5.7%

FEMALES

59

100% 100% 100% 100%

EXPRESSION LEVEL

8.9%

20

25

TABLE IV

EXPRESSION DATA PER LITTER FOR TRANSGENIC PIGS CARRYING THE "185" CONSTRUCT						
Founder	Litter No.	Gilt	Pigs	% Positive	#Tg	Avq. Authentic HbA
38-4	1	544	10	20.0%	2	8.8%
	2	213	11	45.4%	5	4.9%
	3	882	5	20.0%	1	10.9%
	4	4923	6	83.3%	5	9.4%
10.	5	710	6	75.0%	4	4.5%
	6	978	11	36.4%	4	7.1%
	7	466	4	25.0%	1	3.6%
	8	464	15	33.3%	5	5.1%
15	9	461	8	62.5%	5	6.6%
	10	1657	10	30.0%	3	9.0%
	11	892	3	33.3%	1	5.7%
	12	995	11	27.3%	3	4.4%
20	13	209	11	36.4%	4	5.4%
	14	424	10	30.0%	3	5.9%
	15	1659	14	35.7%	5	4.4%
	16	420	12	8.3%	1	2.0%
25	17	373	7	28.6%	2	11.8%
	18	497	8	62.5%	5	6.0%

TABLE IV (CONT'D)

EXPRESSION DATA PER LITTER FOR TRANSGENIC PIGS CARRYING THE "185" CONSTRUCT						
Founder	Litter No.	Gilt	Pigs	% Positive	#Tg	Avg. Authentic HbA
5	19	742	8	25.0%	2	1.0%
	20	1420	14	42.9%	6	8.1%
	21	41	5	40.0%	2	1.0%
	22	540	11	36.4%	4	5.3%
	23	7114	11	54.5%	6	3.4%
10	24	744	11	27.3%	3	4.9%
	25	600	14	42.9%	6	5.5%
	26	1180	9	44.4%	4	2.0%
	27	1137	12	25.0%	3	6.1%
	28	970	8	37.5%	3	10.8%
15	29	78	6	0	0	
	30	214	14	50.0%	7	5.5%
	31	279	6	50.0%	3	10.3%
	32	281	11	45.5%	5	5.1%
	33	21-474	6	33.3%	2	12.3%
20	34	1151	10	30.0%	3	5.3%
			318		118	



# TABLE V

## 38-4 BREEDING SUMMARY

10	FOUNDER				118	FEMALES		59	MALES	
	LITTERS	PIGLETS	PIGS/LITTER	TRANSGENIC		FREQUENCY	AVG. AUTHENTIC HbA	EXPRESSSION LEVEL	EXPRESSSION LEVEL	EXPRESSSION LEVEL
	34	318	9.4	118	37.1%	6.2%		5.7%	6.8%	

15 MALES

20

25

The birth weights of the transgenic pigs have been approximately equivalent to the birth weights of their non-transgenic littermates. As the transgenic pigs matured, their weights remained comparable to the weights of control animals.

## 7. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY DEAE CHROMATOGRAPHY

### 7.1. MATERIALS AND METHODS

#### 7.1.1. PURIFICATION BY DEAE CHROMATOGRAPHY

For purification, red blood cells were collected by centrifugation of 5000 rpm for 3 minutes in an eppendorf microcentrifuge and washed three times with an equal volume (original blood) of 0.9% NaCl.

Red cells were lysed with 1.5 volumes deionized H<sub>2</sub>O, centrifuged at 15,000 rpm, and the supernatant was fractionated by anion exchange chromatography. DEAE cellulose chromatography (DE-SE manufactured by Whatman, Ltd.) was performed according to W. A. Schroeder and T. H. J. Huisman "The Chromatography of Hemoglobin", Dekker, New York, pp. 74-77. The 0.25 ml red cell hemolysate described above was applied to 1 cm x 7 cm DE-52 column pre-equilibrated in 0.2 M glycine Ph 7.8 and was washed with 5 column volumes of 0.2 M glycine Ph 7.8/5 mM NaCl. Hemoglobins were eluted with a 200 ml 5-30 mM NaCl/0.2 M glycine pH 7.8 gradient. To complete elution of pig hemoglobin, an additional 50 to 100 ml of 30 mM CaCl<sub>2</sub>/glycine pH 7.8 was added to the column. Elution of hemoglobin was monitored by absorbance of 415 mμ and by IEF analysis of column fractions.

#### 7.1.2. REASSOCIATION OF GLOBIN CHAINS

Reassociation of globin chains was performed essentially as described in Methods in Enzymol. 76:126-133. 25 lambda of pig blood, 25 lambda of

human blood, or a 25 lambda mixture of 12.5 lambda human blood and 12.5 lambda pig blood were treated as follows. The blood was pelleted at a setting of 5 on microfuge for 2 minutes, then washed three times with 100 lambda 0.9 percent NaCl. The cells were lysed with 50 lambda H<sub>2</sub>O, then spun at high speed to confirm lysis. 50 lambda of the lysed cells was then combined with 50 lambda 0.2 M Na Acetate, pH 4.5, put on ice and then incubated in a cold room overnight. After adding 1.9 ml 0.1 M NaH<sub>2</sub>PO<sub>4</sub> pH 7.4 each sample was spun in centricon tubes at 4°C and 5K until about 0.5 ml remained. Then 1 ml of 0.1 M NaH<sub>2</sub>PO<sub>4</sub> pH 7.4 was added and spun through at about 5K until about 0.2 ml volume was left. The hemoglobin was then washed from the walls of the centricon tube, an eppendorf adaptor was attached, and a table top microfuge was used to remove each sample from its centricon tube. The samples were then analyzed by isoelectric focusing.

## 7.2. RESULTS AND DISCUSSION

### 7.2.1. HUMAN AND PIG HEMOGLOBIN WERE SEPARATED FROM A HEMOLYZED MIXTURE OF HUMAN AND PIG BLOOD

Equal proportions of human and of pig blood were mixed and lysed, and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4A, pig hemoglobin separated virtually completely from human hemoglobin. This complete separation is surprising in light of the structural similarity between human and pig hemoglobin; pig and human alpha globin chains are 84.4 percent homologous and pig and human beta globin chains are 84.9 percent homologous. It is further surprising because, as shown in Figure 4C, when human and mouse blood was mixed, hemolyzed, applied to and eluted from a DEAE column according to methods set forth in Section 7.1.1. supra, human and mouse

hemoglobin were not observed to separate despite the fact that mouse and human alpha globin chains are about 85.8 percent homologous and mouse and human beta globin chains are 80.1 percent homologous. The ease of separation of human and pig hemoglobin on DEAE resin appears to be both efficient and economical.

Interestingly, the order of elution of the proteins from the anion exchange column was not as expected. Based on the relative pI's of the proteins as deduced from the IEF gels, the predicted order of elution would be first the hybrid (human  $\alpha$ /pig  $\beta$ ) followed by the authentic human  $\alpha$ /human  $\beta$ . The last protein to elute from the anion exchange column then would be the endogenous pig  $\alpha$ /pig  $\beta$  protein. However, under all the conditions currently attempted the order of elution was altered such that the human hemoglobin was the first to elute. The second peak was an enriched fraction of the hybrid followed very closely by the pig hemoglobin.

7.2.2. HUMAN AND PIG HEMOGLOBIN AND HUMAN/PIG HETEROLOGOUS HEMOGLOBIN WERE SEPARATED FROM HEMOLYSATE PREPARED FROM A TRANSGENIC PIG

Blood from transgenic pig 6-3 (as described in Section 6, supra) was lysed by hypotonic swelling and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4B, human hemoglobin was separated from pig hemoglobin and from human  $\alpha$  globin/pig beta globin heterologous hemoglobin. As shown in Figure 4D, human hemoglobin was substantially purified by this method.

7.2.3. PIG ALPHA GLOBIN/HUMAN BETA GLOBIN HETEROLOGOUS HEMOGLOBIN DOES NOT APPEAR TO FORM BASED ON REASSOCIATION DATA

Heterologous association between pig alpha globin and human beta globin chains has not been detected in hemolysates obtained from human hemoglobin-expressing transgenic pigs. It was possible, however, that this observation could be explained by relatively low levels of human beta globin expression. Alternatively, association between pig alpha globin and human beta globin may be chemically unfavorable. In order to explore this possibility, reassociation experiments were performed in which pig and human hemoglobin were mixed, dissociated, and then the globin chains were allowed to reassociate. As shown in the isoelectric focusing gels depicted in Figure 5, although pig  $\alpha$ /pig  $\beta$ , human  $\alpha$ /human  $\beta$ , and human  $\alpha$ /pig  $\beta$  association was observed, no association between pig  $\alpha$  globin and human  $\beta$  globin appeared to have occurred. Therefore the pig  $\alpha$ /human  $\beta$  heterologous hemoglobin should not be expected to complicate the purification of human hemoglobin from transgenic pigs.

#### 8. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY QCPI CHROMATOGRAPHY

##### 8.1. MATERIALS AND METHODS

Clarified hemolysate from transgenic pig 6-3 13mg/ml; Buffer A: 10mM Tris, 20mM Glycine pH 7.5; Buffer B: 10mM Tris, 20mM Glycine, 15 mM NaCl pH 7.5; Buffer C: 10mM Tris, 20mM Glycine, 1M NaCl pH 7.5; Buffer D: 10mM Tris, 20mM Glycine, 50 mM NaCl pH 7.5; QCPI column 10ml Equilibrated in Buffer A; Trio purification system. 10mg of hemoglobin prepared from transgenic pig 6-3 was diluted in 20ml Buffer A. 20ml of sample was loaded at a flow rate of 5ml/min onto the QCPI column, and washed with 2 column volumes of Buffer A. The column was then washed with 20 column volumes of a 0-50mM NaCl gradient. (10 column volumes

Buffer A + 10 column volumes of Buffer D) and the O.D.<sub>280</sub> absorbing material was collected. The column was then cleaned with 2 column volumes of Buffer C, and then re-equilibrated with 2 column volumes of

5 Buffer A.

10

11 8.2. RESULTS

12 Reference Analysis of the UV trace (peak vs. volume of gradient) (Fig. 6) revealed that the human hemoglobin

13 was eluted at 15 mM NaCl. Subsequent purifications have been performed utilizing the same protocol as above, only using 6 column volumes of Buffer B (15mM NaCl) to elute the human hemoglobin rather than the gradient. In addition, non-transgenic pig

14 chromatographed by this method does not elute from the QCPI with Buffer B, while native human hemoglobin does. The protein that eluted at 15mM NaCl was analyzed on the Resolve isoelectric focussing system and found to be essentially pure of contaminating pig

15

20 hemoglobin or hybrid hemoglobin.

#### 9. EXAMPLE: HUMAN ALPHA/PIG BETA GLOBIN HYBRID HEMOGLOBIN EXHIBIT INCREASED P<sub>50</sub>

As shown in Tables II and III, supra,

25 transgenic pigs of the invention were all found to produce significant amounts of human  $\alpha$ /pig  $\beta$  globin hybrid hemoglobin (the pig  $\alpha$ /human  $\beta$  hybrid was not observed). Significantly, pigs that expressed higher percentages of hybrid also appeared to exhibit

30 elevated P<sub>50</sub> values for their whole blood (Figure 7).

#### 10. EXAMPLE: ENHANCED EXPRESSION USING PIG BETA GLOBIN REGULATORY SEQUENCES

The 339 construct (Figures 1R and 12)

35 containing the pig adult beta globin gene promoter region (Figure 8), was used to prepare transgenic pigs

according to the method set forth in Section 6.1.2.  
supra. Figure 15 depicts an isoelectric focusing gel  
analysis of hemoglobin produced by pig 70-3; equal  
amounts of hemoglobin from transgenic pig 6-3,  
5 carrying the 116 construct (Figure 1A) and human  
hemoglobin are run in adjacent lanes for comparison.  
As indicated by the brighter bands observed in the  
lane containing pig 70-3 hemoglobin at positions  
corresponding to human and hybrid hemoglobins  
10 (relative to the lane containing pig 6-3 hemoglobin),  
the amount of human hemoglobin produced by pig 70-3 is  
greater than the amount produced by pig 6-3. It has  
been calculated that 38 percent of the total  
hemoglobin produced by pig 70-3 is human hemoglobin,  
15 whereas 10 percent of total hemoglobin produced by pig  
6-3 is human hemoglobin (see Table II and Section 6.2.  
supra, for data and calculations). This suggests that  
the pig beta globin promoter region is more efficient  
than the human beta globin promoter in transgenic  
20 pigs.

In a separate series of experiments, two  
more transgenic pigs, expressing human hemoglobin,  
were obtained using construct "339" (pigs 80-4 and 81-  
3) (FIG. 17). Human hemoglobin levels in these  
25 transgenic pigs was determined by running isoelectric  
focussing gels and densitometric scanning of the  
individual bands (FIG. 18). As indicated in Figure  
17, both pig 70-3 and pig 80-4 expressed high levels  
of authentic human hemoglobin. To obtain the copy  
30 number of transgenes, genomic DNA (isolated from the  
tail) was digested with EcoR I and a Southern Blot was  
performed. The probe used was a 427 bp NcoI/Bam HI  
fragment of human beta globin gene containing the  
first exon, first intron and part of the second exon.

35

11. EXAMPLE: MOLECULAR MODELING OF PIG  
HEMOGLOBIN AND THE  $\alpha_1$   $\beta_1$  INTERFACE OF

A HYBRID BETWEEN PIG  $\beta$  AND HUMAN  $\alpha$  GLOBIN

It has been found that the amount of hybrid human  $\alpha$ /pig  $\beta$  hemoglobin often exceeds the amount of human hemoglobin. The molecular basis of this observation has been investigated using molecular modeling and molecular biology. The model structure of the hybrid molecule is based on the known structures of human hemoglobins and the structural homology between the human and pig structures (A.M. Lesk, 1991, Protein Architecture: A Practical Approach, Oxford University Press, N.Y.). The pig and hybrid hemoglobin structures were modeled using the following four steps: (1) hydrogen atoms were added to the X-ray model and their positions modified using energy minimization; (2) amino acid residue replacements were introduced to model the target pig and hybrid structures (no chain alignment was necessary); (3) the side chain positions of these modified residues were energy minimized; and (4) the result was visually examined and found to be sound. The modeled structures are shown in Figure 20.

Detailed examination of all the relevant contacts indicated striking differences at several residues. For example, at position  $\beta 112$  the human hemoglobin has a cysteine residue but the hybrid has a valine residue. The valine is in apparent closer contact (arrow in FIG. 20) with the opposing subunit, and thus may be more effective in stabilizing the  $\alpha_1 \beta_1$  interface (FIG. 21).

The effect of amino acid substitutions at the  $\alpha_1 \beta_1$  interface on the hydrophobic and polar interactions as predicted by HINT are shown in TABLE VI. HINT is software from Virginia Commonwealth University Licensed from Medical College of Virginia, Richmond, Virginia that can analyze the positive and negative scores as determined by attractive and



repulsive interactions known from experimental physical chemistry measurements. TABLE VI represents the differences between the unmodified dimer and the one with the specified replacement. TABLE VII has the same format as TABLE VI with the following two exceptions: (1) as each replacement is added, the previous one(s) are kept, and (2) the reported difference is a comparison between the current dimer and the one reflected in the preceding row. As the subsequent changes are made, the predicted attractive forces at the interface increase. If each column is summed up the total difference between the unmodified dimer and the one with seven changes is obtained. The sums are +1340 for hydrophobic and +660 for polar.

12.1. REPRODUCTION and growth of the organism  
of the plant or animal

[illegible]

TABLE VI

Effect of amino acid replacements at the  $\alpha 1\beta 1$  interface

	Chain	Residue	Replacement	Predicted Difference	
				Hydrophobic	Polar
10	$\alpha$	30	E to T	+250	+10
	$\alpha$	36	F to Y	-110	+220
	$\alpha$	106	L to F	+20	+10
15	$\alpha$	107	V to S	-10	+120
	$\alpha$	107	V to C	0	+150
	$\alpha$	111	A to C	+30	+100
20	$\beta$	33	V to L	+70	0
	$\beta$	112	C to V	+330	-60
	$\beta$	112	C to I	+360	-50
	$\beta$	115	A to V	+80	+10
25	$\beta$	115	A to L	+90	+10
	$\beta$	119	G to H	+250	+120
	$\beta$	125	P to M	+80	0
	$\beta$	128	A to I	+80	0
30	$\beta$	131	Q to E	+120	+110

**TABLE VII**  
**Effect of combinations of amino acid replacements**  
**at the  $\alpha 1\beta 1$  interface on the hydrophobic and polar interactions**

Predicted Difference

<u>Chain</u>	<u>Residue</u>	<u>Replacement</u>	<u>Hydrophobic</u>	<u>Polar</u>
$\beta$	112	C to I	+360	-50
$\alpha$	110	A to I	+200	+10
$\beta$	115	A to V	+150	+10
$\beta$	119	G to H	+270	+130
$\alpha$	36	F to Y	-130	+240
$\beta$	33	V to L	+80	+0
$\alpha$	30	E to T	+260	+10
$\beta$	131	Q to E	+150	+310

12. EXAMPLE: EXPRESSION OF GENETICALLY  
MODIFIED HEMOGLOBINS IN TRANSGENIC ANIMALS

Of the known human hemoglobin variants,  
5 about two dozen exhibit a lower oxygen affinity, which  
could be advantageous in clinical applications. While  
many of these mutants result in unstable hemoglobin  
molecules, several variants have desirable biochemical  
properties and can be used for the generation of blood  
10 substitutes using recombinant DNA technology.  
Transgenic pigs expressing two of these variants, Hb  
Presbyterian (108 Asn→Lys, Fig. 1G) and Hb Yoshizuka  
(108 Asn→Asp, Fig. 1F) have been produced and  
purification and characterization of the expressed  
15 human globins is described below.

12.1. PURIFICATION AND CHARACTERIZATION  
OF Hb PRESBYTERIAN

The amino acid substitution generated in Hb  
Presbyterian ( $\beta$ 108 Asn→Lys) results in the comigration  
20 of Hb Presbyterian with the hybrid (hap $\beta$ ) hemoglobin  
on isoelectric focussing gels. Based on previous  
results with the purification of human hemoglobin from  
hybrid and porcine hemoglobins and the more positive  
nature of the Hb Presbyterian it should be easier to  
25 purify this variant hemoglobin on an anion exchange  
resin. Approximately 500 ml of blood was obtained  
from the transgenic pig 57-10. The blood was washed  
several times with isotonic saline and then lysed by  
hypotonic swelling in water. The cell membranes were  
30 removed by centrifugation at 10000 xg to yield a final  
hemoglobin concentration of about 100 mg/ml. Hb  
Presbyterian was purified from the hybrid and porcine  
hemoglobins as follows: 1-2.5 g of hemolysate was  
loaded onto an XK 50/30 column packed with 450 ml of  
35 Biorad Macrorep High Q resin equilibrated with 10 mM  
Tris-Cl and 20 mM Glycine at pH 8.1 (Buffer A). The

proteins were eluted at a flow rate of 10 ml/min with a linear salt gradient of 9-16% Buffer B (Buffer A containing 250 mM NaCl) over 3000 ml.

- The initial peak was thought to be Hb Presbyterian followed by the co-elution of the hybrid and porcine hemoglobins (FIG. 20). To confirm the identity of the first peak as Hb Presbyterian and not the hybrid hemoglobin, a sample of the protein was run on Reversed Phase HPLC (FIG. 21). The initial peak from the anion exchange column was Hb Presbyterian with the  $\alpha$ -chains eluting at the same time as normal human  $\alpha$ -chains and the  $\beta$ -chains eluting slightly faster than normal human  $\beta$ -chains. This was also found to be an excellent way of determining if porcine hemoglobin was contaminating the column fractions. Using this purification procedure and the analysis on HPLC the recombinant Hb Presbyterian derived from the transgenic pig 58-10 was judged to be greater than 95% pure.
- Purified Hb Presbyterian was dialyzed against 50 mM HEPES and 100 mM NaCl at pH 7.4 and oxygen equilibrium curves determined using a Hemox Analyzer (TCS Products, Southampton, PA). The Hemox Analyzer was modified to allow analog to digital data conversion for ease of oxygen binding calculations. Under these conditions the Hb Presbyterian had a  $P_{50}$  of 25.8 mmHg (Hill Coefficient  $n=2.3$ ) versus 13.3 mm Hg ( $n=2.9$ ) for Hb A indicating that the Hb Presbyterian bound oxygen with lower affinity than native Hb.
- Preliminary results to determine the Bohr Effect (Influence of pH on the oxygen affinity) indicated a normal Bohr effect for Hb Presbyterian (FIG. 22).

## 12.2. PURIFICATION AND CHARACTERIZATION OF Hb YOSHIZUKA

Blood samples taken from the transgenic pigs expressing Hb Yoshizuka (68-3 and 68-2) were treated essentially the same as described above. The final concentration of the hemolysate was approximately 100 mg/ml. The purification of the protein required a slightly different strategy, however. A sample of hemolysate from 68-3 (about 10 mg) was loaded onto an HR 10/30 Biorad Macroprep High Q resin column equilibrated with 10 mM Tris-Cl and 20 mM Glycine at pH 8.7 (Buffer A). The hemoglobins were eluted at 2.5 mls/min with a 5-30% linear gradient of Buffer B (Buffer A plus 250 mM NaCl) over 500 ml (FIG. 23). Fractions were collected and analyzed by IEF to assess purity which was determined to be about 75% or better.

## 13. DEPOSIT OF MICROORGANISMS

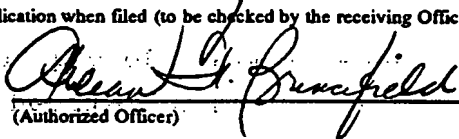
The following plasmids were deposited with the American Type Culture Collection (ATCC) 12301, Parklawn Drive, Rockville, Maryland 20852 on December 2, 1992.

plasmid containing the accession no.

psaf/pig $\epsilon$ (k)	pig $\epsilon$ globin gene	75371
pGem5/Pig $\beta$ pr(K)	pig adult $\beta$ globin gene regulatory region	75372
pPig3' $\beta$	3'-end of pig $\beta$ globin gene	75373

Various publications are cited herein which are hereby incorporated by reference in their entirety.

International Application No: PCT/

<b>MICROORGANISMS</b>	
Optional Sheet in connection with the microorganism referred to on page 52, lines 18-28 of the description *	
<b>A. IDENTIFICATION OF DEPOSIT</b> * Further deposits are identified on an additional sheet.	
Name of depositary institution * <b>American Type Culture Collection</b>	
Address of depositary institution (including postal code and country) * <b>12301 Parklawn Drive Rockville, MD 10582 US</b>	
Date of deposit * <b>December 2, 1992</b> Accession Number * <b>75371</b>	
<b>B. ADDITIONAL INDICATIONS</b> * (leave blank if not applicable). This information is obtained on a separate attached sheet <input checked="" type="checkbox"/>	
<p>1. Instead of the word "strain" or "strain" instead of "val" at position 111.</p>	
<b>C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> * (if the indications are not all designated, State)	
<p>The word "strain" or "strain" instead of "val" at position 111.</p>	
<b>D. SEPARATE FURNISHING OF INDICATIONS</b> * (leave blank if not applicable)	
<p>The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")</p> <p>1. Instead of the word "strain" or "strain" instead of "val" at position 111.</p>	
<p><b>E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office)</b></p> <p style="text-align: right;">         (Authorized Officer)     </p> <p><input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau *</p> <p style="text-align: right;">       was _____        (Authorized Officer)     </p>	

International Application No: PCT/

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive  
Rockville, MD 10582  
US

Accession No.

75372

75373

Date of Deposit

December 2, 1992

December 2, 1992

24



## WHAT IS CLAIMED IS:

1. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the 426 construct as depicted in Figure 14.
2. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the 427 construct as depicted in Figure 14.
3. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the amount of human globin produced relative to total hemoglobin is at least twenty percent.
4. A transgenic pig comprised of a DNA sequence comprising the pig adult  $\beta$  globin regulatory region as contained in plasmid pGem5/Pig $\beta$ pr(K), deposited with the American Type Culture Collection and assigned accession number 75371, operably linked to a gene, in which the gene does not encode pig adult  $\beta$  globin, where the gene is expressed in at least some of the red blood cells of said pig.
5. The transgenic pig of claim 4 in which the gene is human  $\beta$  globin.

6. The transgenic pig of claim 4 in which the gene encodes a non-globin protein.

7. A transgenic pig comprised of a DNA sequence comprising the 3' region of the pig adult  $\beta$  globin gene, as contained in plasmid pPig3' $\beta$ , deposited with the American Type Culture Collection and assigned accession number 75372, operably linked to a gene, in which the gene is not pig adult  $\beta$  globin, where the gene is expressed in at least some of the red blood cells of said pig.

8. The transgenic pig of claim 7 in which the gene is human  $\beta$  globin.

9. The transgenic pig of claim 7 in which the gene encodes a non-globin protein.

10. A purified and isolated nucleic acid comprising: the pig adult  $\beta$  globin regulatory region as comprised in plasmid pGem5/Pig $\beta$ pr(K), as deposited with the American Type Culture Collection and assigned accession number 75371.

11. A purified and isolated nucleic acid comprising: the pig  $\epsilon$  globin gene as comprised in plasmid pSaf/pig $\epsilon$ (K), as deposited with the American Type Culture Collection and assigned accession number 75373.

12. A purified and isolated nucleic acid comprising: the 3' region of the pig adult  $\beta$  globin gene as comprised in plasmid pPig3' $\beta$ , as deposited with the American Type Culture Collection and assigned accession number 75372.

13. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid encoding human alpha globin or human beta globin comprises a mutation which increases the level of authentic human/human dimer in the transgenic pig.

10 14. The transgenic pig of claim 13 wherein the mutation in human alpha hemoglobin is selected from the following group of alpha-chain mutations: a Thr at position 30 instead of Glu; a Tyr at position 36 instead of Phe; a Phe instead of Leu at position 106; a Ser or Cys instead of Val at position 107; and a Cys instead of Ala at position 111.

15 15. The transgenic pig of claim 13 wherein the mutation in human beta hemoglobin is selected from the following group of beta-chain mutations: a Leu instead of Val at position 33; a Ile instead of Cys at position 112; a Val or Leu instead of Ala at position 115; a His instead of Gly at position 119; a Met instead of Pro at position 128; and a Glu instead of Gln at position 131.

16. The transgenic pig of claim 15 wherein the mutation in human beta hemoglobin is a Cys to Val change at position 112.

30

17. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and in which the nucleic acid construct is the hemoglobin Presbyterian construct as depicted in Figure 1G.

18. A method for purifying human Presbyterian Hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

- 5 (i) collecting red blood cells from a transgenic pig according to claim 17;
- (ii) releasing the contents of the collected red blood cells to  
10 produce a lysate;
- (iii) applying the lysate of step (ii) to a High Q resin column equilibrated with 20 mM Tris-Cl and 20 mM Glycine at a pH 8.1;
- 15 (iv) eluting the column with a linear salt gradient of 9-16% in buffer containing 10mM Tris-Cl, 20mM Glycine, 250mM NaCl at pH 8.1; and
- 20 (v) collecting the fractions that contain purified human Presbyterian Hb.

19. A transgenic pig comprised of the DNA sequences encoding human alpha globin and human beta  
25 globin operably linked to promoter elements where human hemoglobin is produced in at least some of the red cells of said pig and The transgenic pig of claim 1 in which the nucleic acid construct is the hemoglobin Yoshizuka construct as depicted in Figure  
30 1F.

20. A method for purifying human Yoshizuka Hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin,  
35 comprising:

- (i) collecting red blood cells from a transgenic pig according to claim 19;
- (ii) releasing the contents of the collected red blood cells to produce a lysate;
- (iii) applying the lysate of step (ii) to a High Q resin column equilibrated with 10mM Tris-Cl and 20mM Glycine at a pH 8.7;
- (iv) eluting the column with a linear salt gradient of 5-30% in buffer containing 10mM Tris-Cl, 20mM Glycine, 250mM NaCl at pH 8.7; and
- (v) collecting the fractions that contain purified human Yoshizuka

2-Phenyl-1,2,3,4-tetrahydro-1H-benzodiazepine-5-carboxamide hydrochloride (clonazepam) HCl

1000000

$\alpha\alpha\beta$

# CONSTRUCT #116

(16.9 kb)



LCR

FIG. 1A

$\alpha$ -Promoter- $\beta$   
CONSTRUCT #185

(13.5 kb)  
CONSTRUCT #185

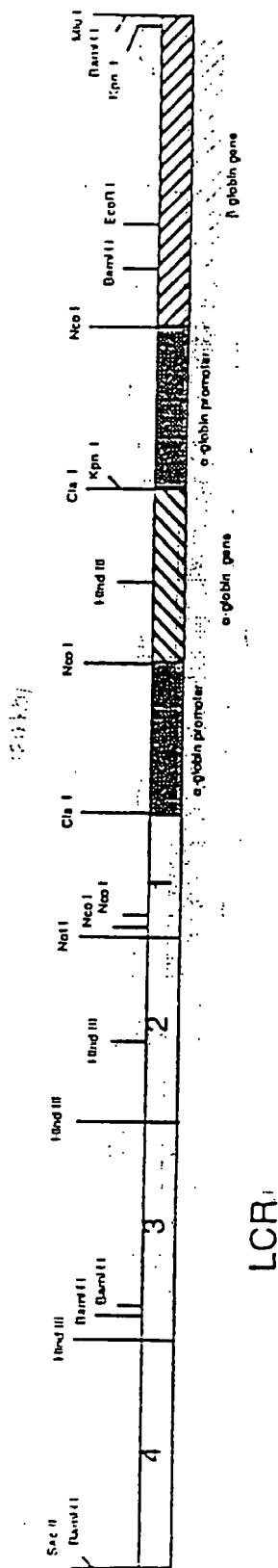


FIG. 18

Hb Eusbovian  
 $\beta$ -Promoter- $\alpha$   
CONSTRUCT #290

(13.9 kb)

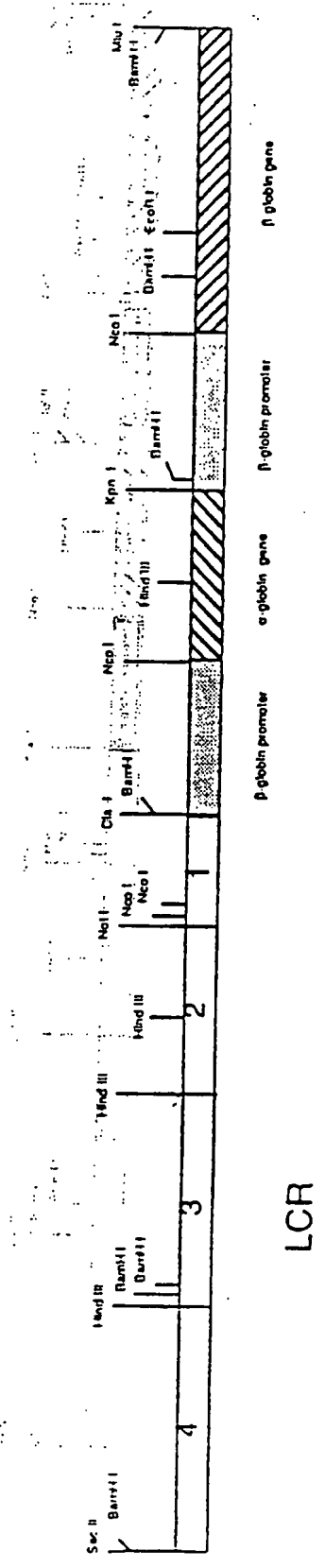


FIG. 1C



Hb Presbyterian  
 CONSTRUCT<sub>epζβα</sub>  
 (20 kb)

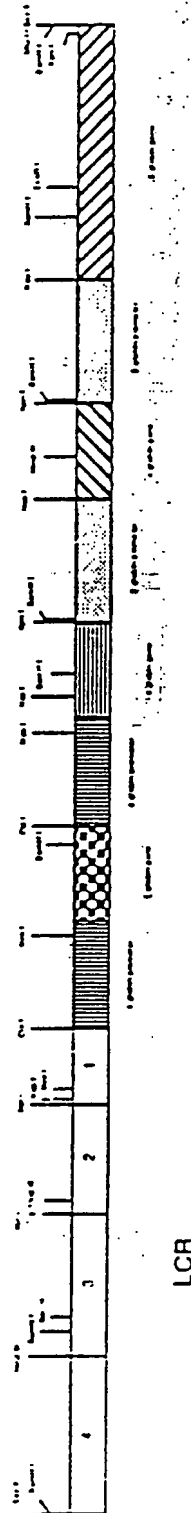


FIG. 1 D

FIG. 1 E

CONSTRUCT #227

CONSTRUCT  $\zeta_{pe\alpha\beta}$

(20 kb)

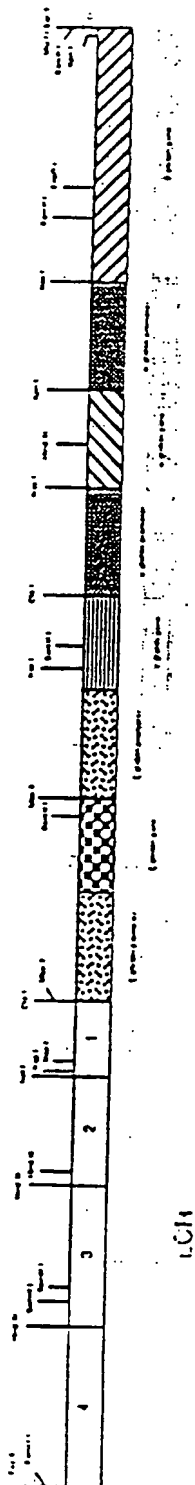


FIG. 1E

# Hb Yoshizuka

$\alpha\beta$

(13.5 kb)

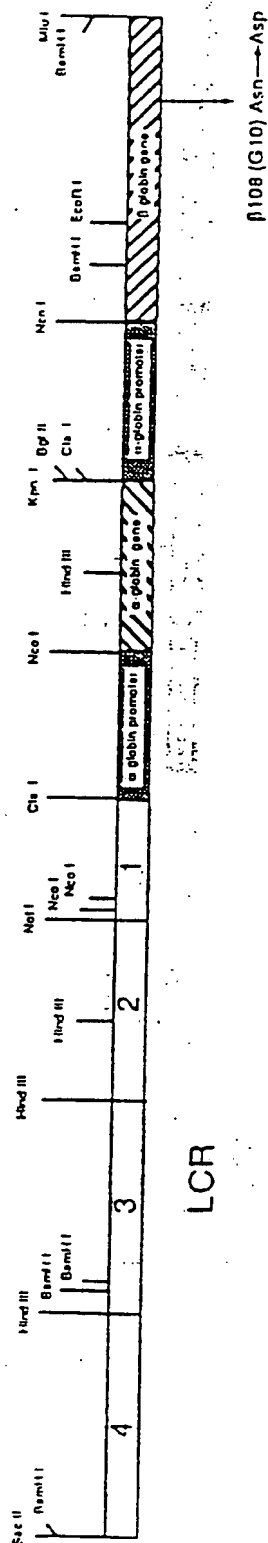


FIG. 1F

FIG. 1

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U-Promoter

CONSTRUCT #293  
Hb Presbyterian

$\alpha\beta$

(13.5 kb)

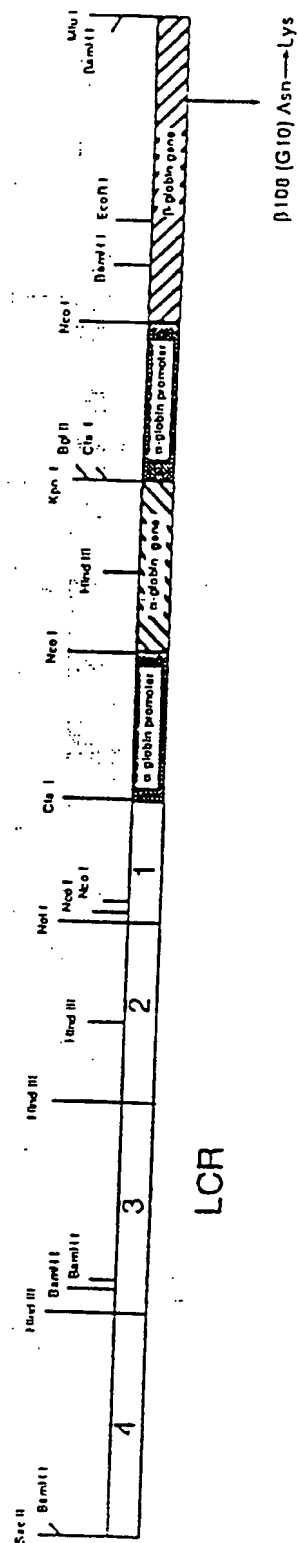


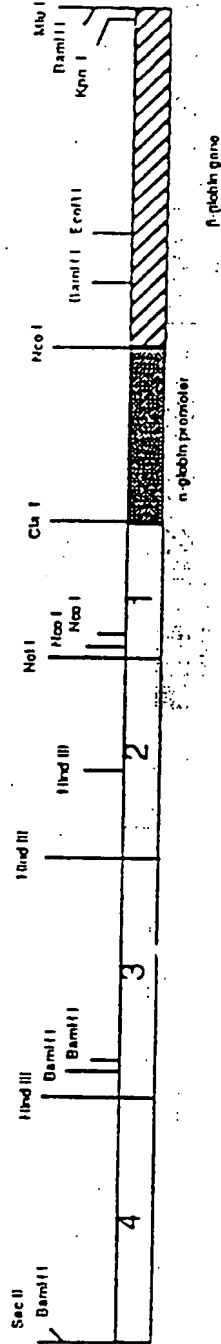
FIG. 1G

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# CONSTRUCT #285

## $\alpha$ -Promoter- $\beta$ ( $\Delta\alpha$ )

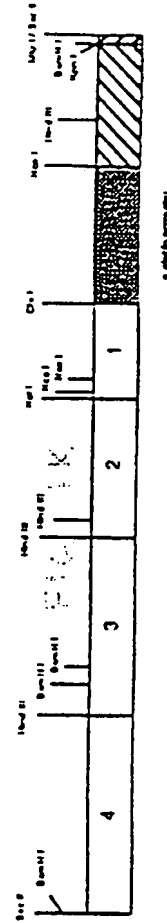
(10.8 kb)



LCR

## LCR $\alpha$

(9.2 kb)



n-globin gene

FIG 1 H

LCR

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CONSTRUCT #240

LCR  $\alpha\beta$

(12.9 kb)

CONSTRUCT #227

$\alpha\beta$

(13.5 kb)

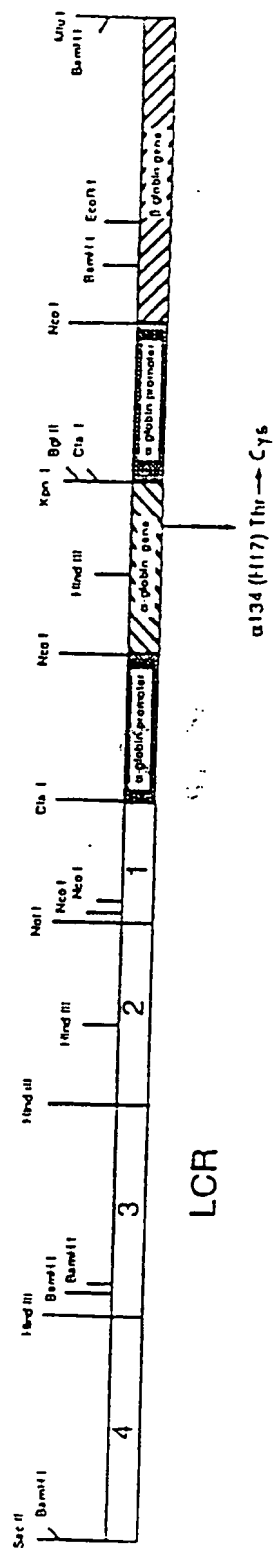


FIG. 1I

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# CONSTRUCT #228

$\alpha\beta$

(13.5 kb)

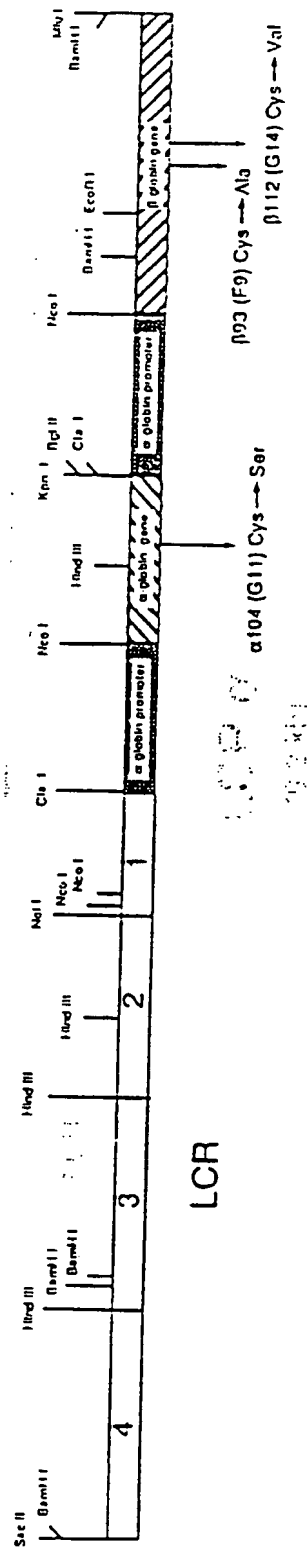


FIG 1J

$\alpha$ -Promoter- $\delta$   
CONSTRUCT #263

(13.1 kb)

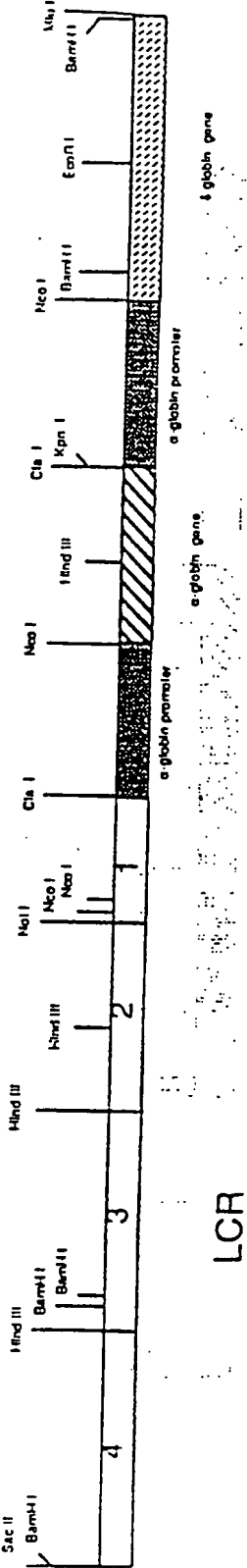
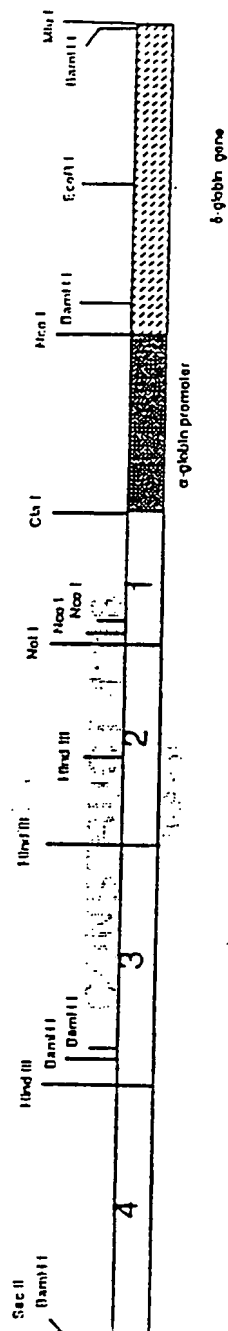


FIG. 1K



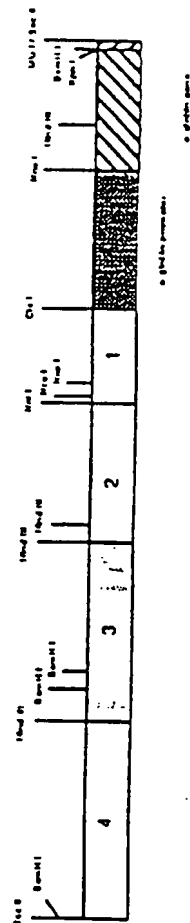
**CONSTRUCT #274**

**$\alpha$ -Promoter- $\delta$  ( $\Delta\alpha$ )**  
(10.4 kb)



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12/52

1472



LCR



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# Hb Bologna

$\alpha\beta$

(13.5 kb)

CONSTRUCT

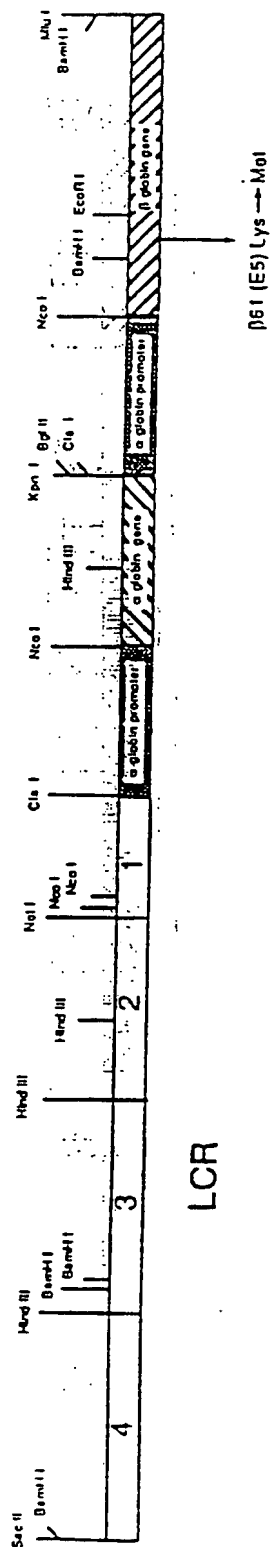


FIG. 1N

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$\epsilon\alpha\beta$

CONSTRUCT #318

(16.9 kb)

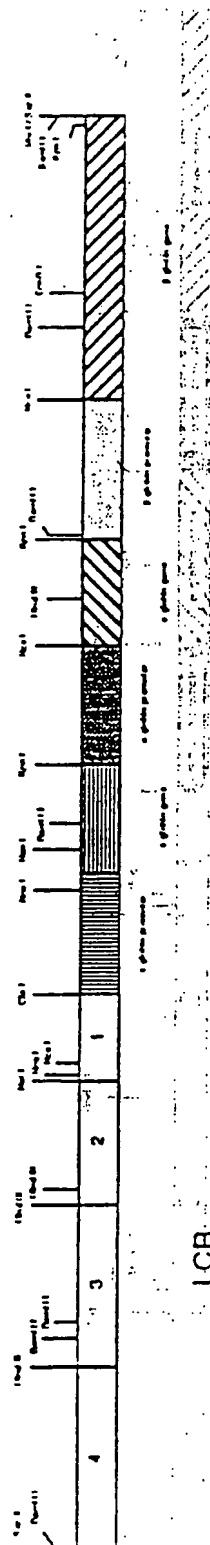


FIG. 10

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$\alpha\epsilon\beta$

CONSTRUCT #319

(16.9kb)

(16.9kb)

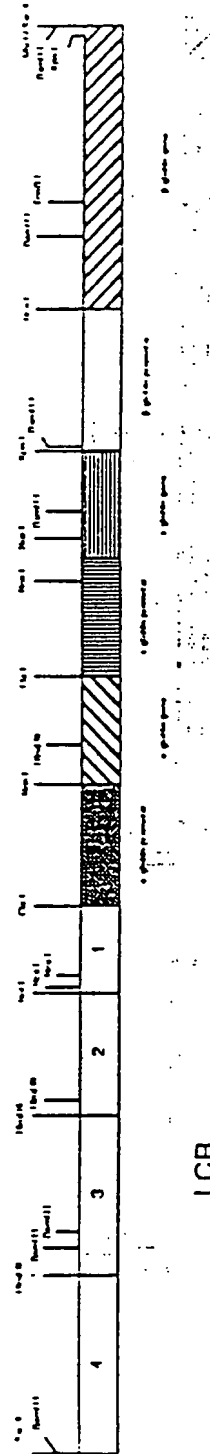


FIG. 1P

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CONSTRUCT #329

CONSTRUCT #329

(20 kb)

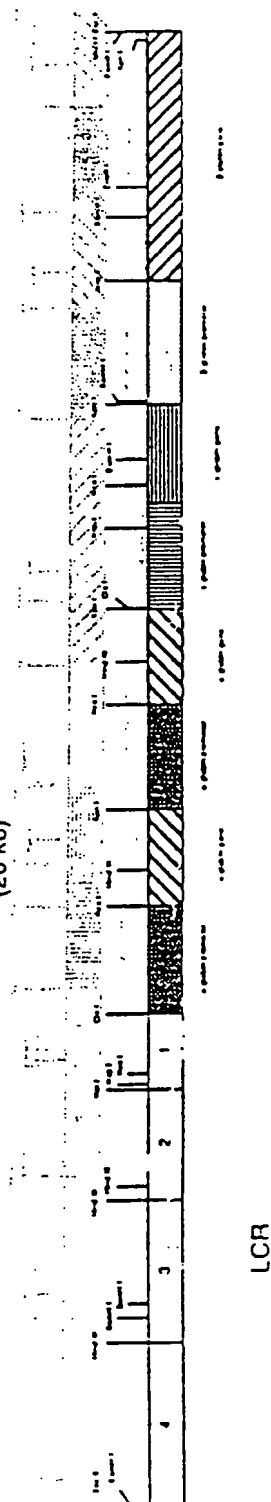


FIG 1Q

$\alpha\epsilon(\text{pig}\beta)\text{p}\beta$   
**CONSTRUCT #339**  
 (18 kb)

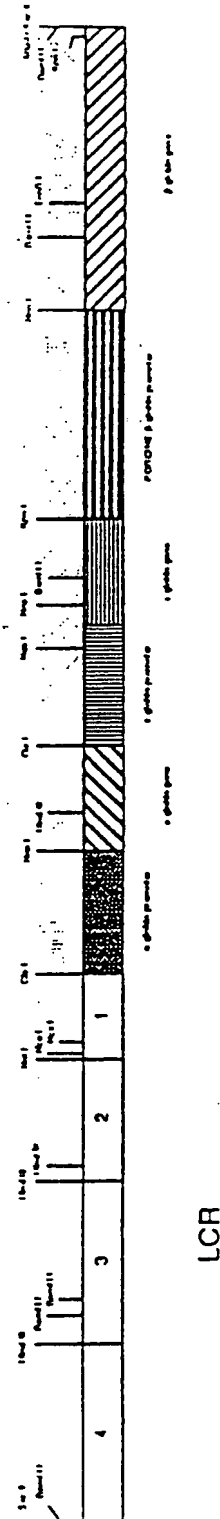


FIG. 1R

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# CONSTRUCT #340

$\alpha\beta$

(13.5 kb)

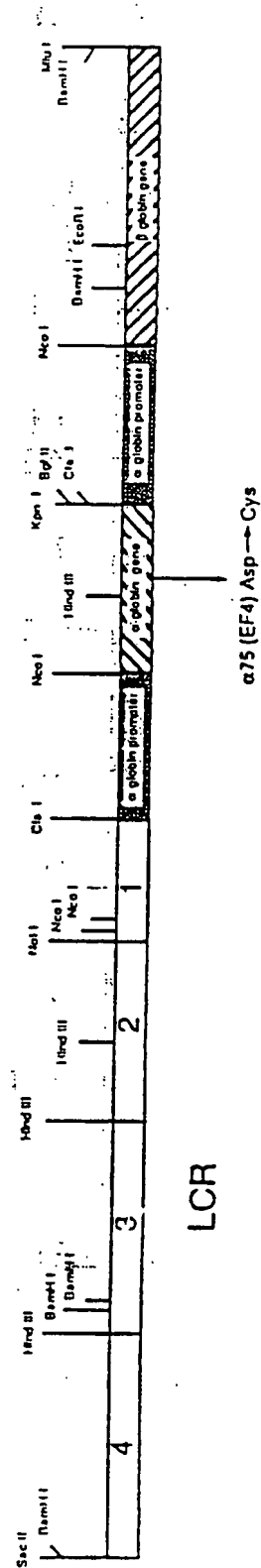


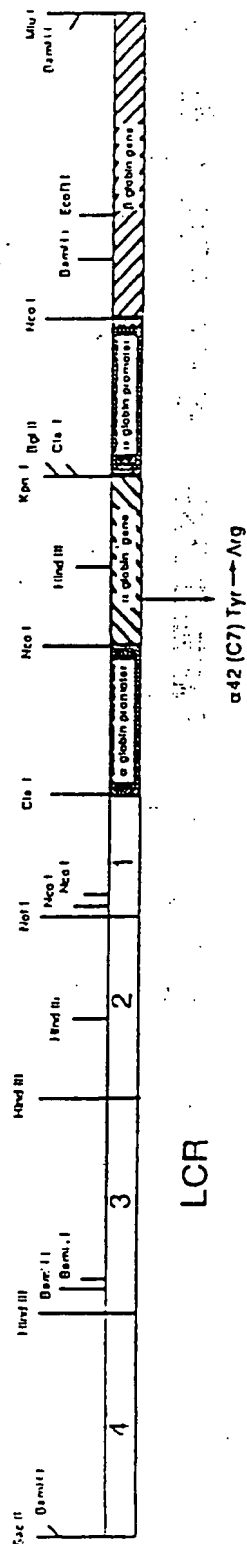
FIG. 1S



**CONSTRUCT #341**

expB

(13.5 kb)



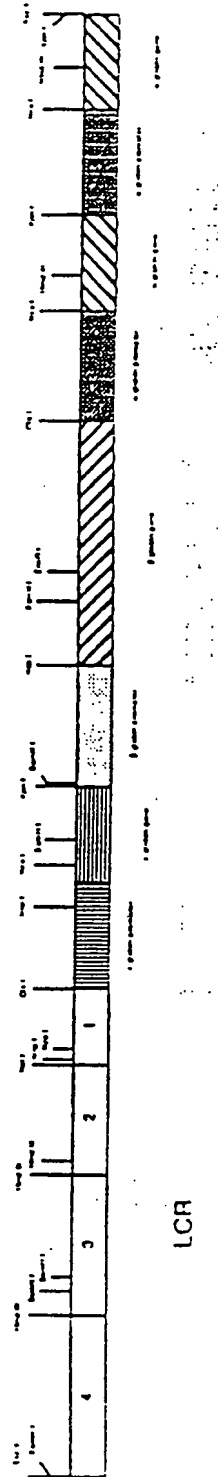
$\epsilon\beta\alpha\alpha$

CONSTRUCT #343

(20 kb)

OPF

10 kb



LCR

FIG. 10

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$\epsilon\beta\alpha$

# CONSTRUCT #347

(16.9 kb)

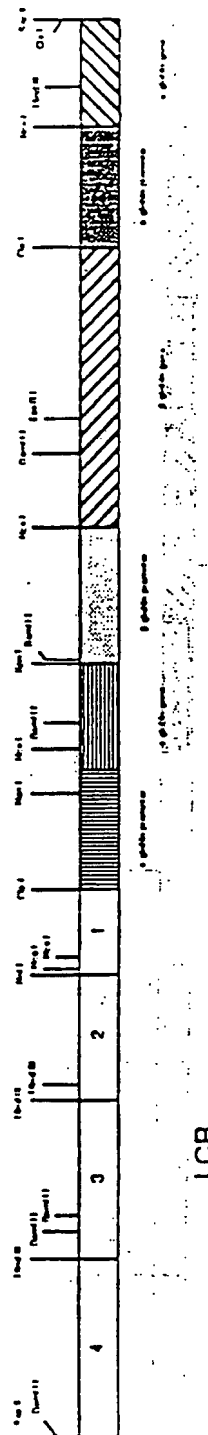


FIG. 1V

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# Alpha 42 Y.K

$\alpha\beta$

(13.5 kb)

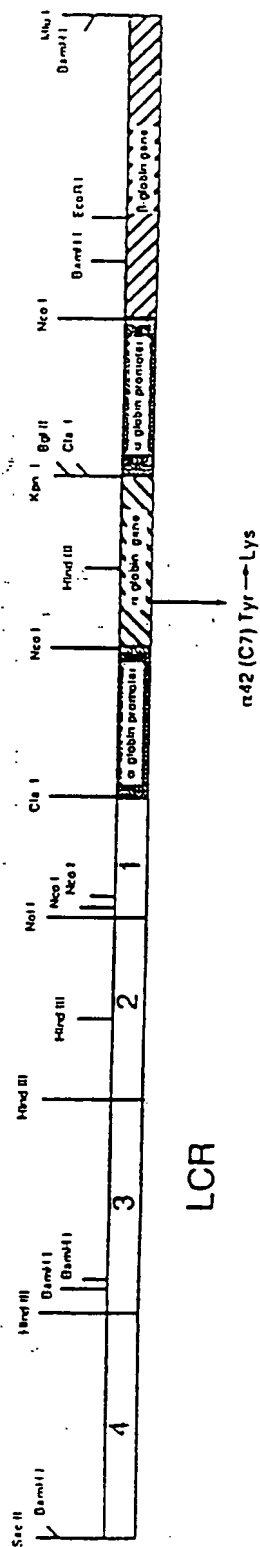


FIG. 1W

$\alpha$  42 Y.R,  $\beta$  99 D.E  
 $\alpha\beta$   
(13.5 kb)

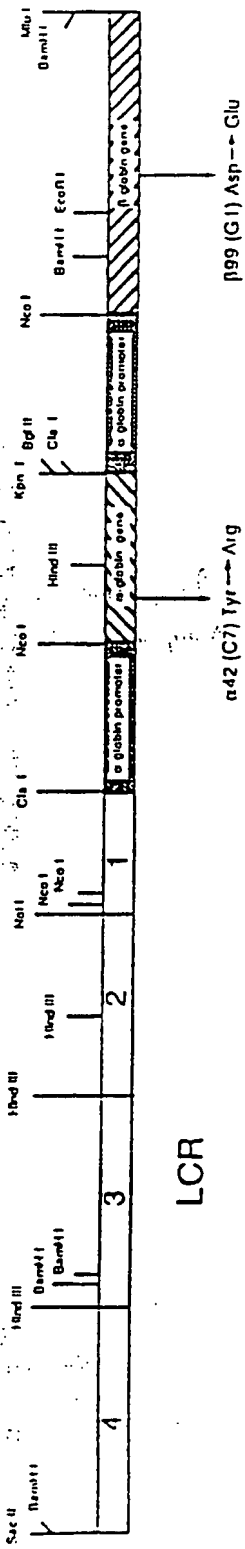


FIG. 1X

$\alpha$  42 Y.K,  $\beta$  99 D.E

$\alpha\beta$

(13.5 kb)

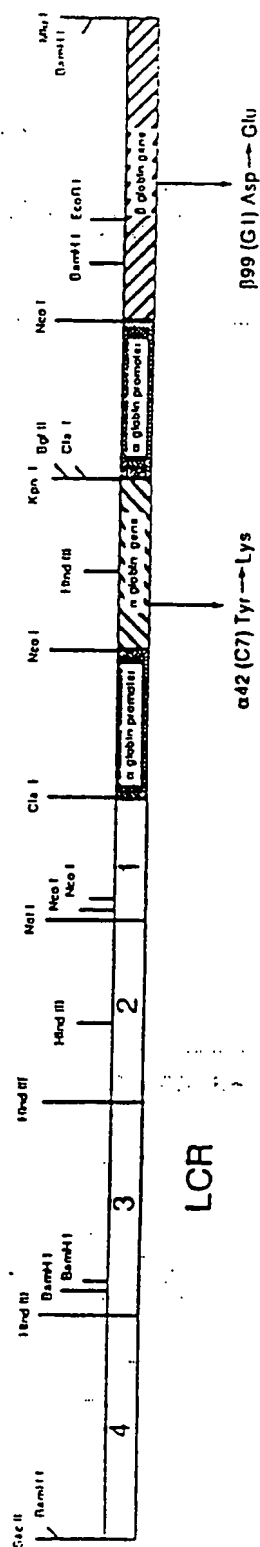


FIG. 1Y

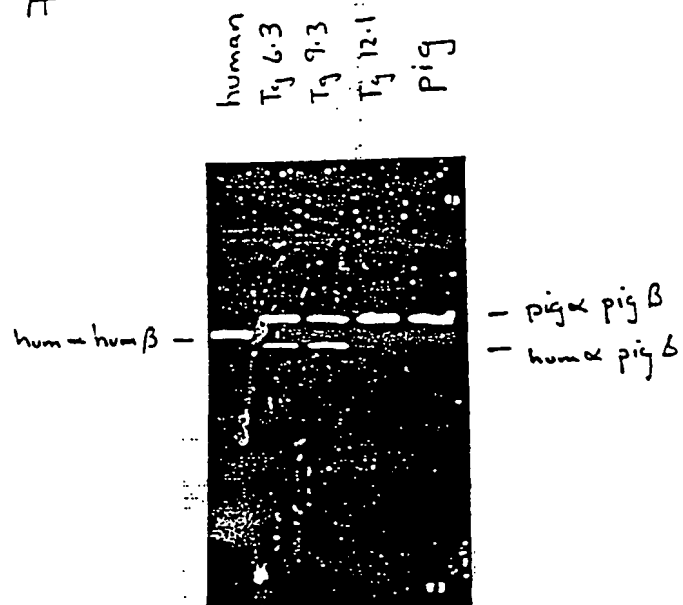


FIG. 2

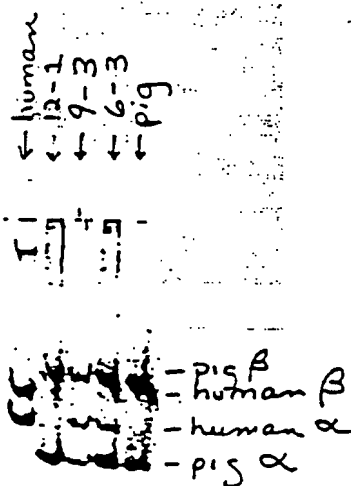
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FIG. 3 A-B

A



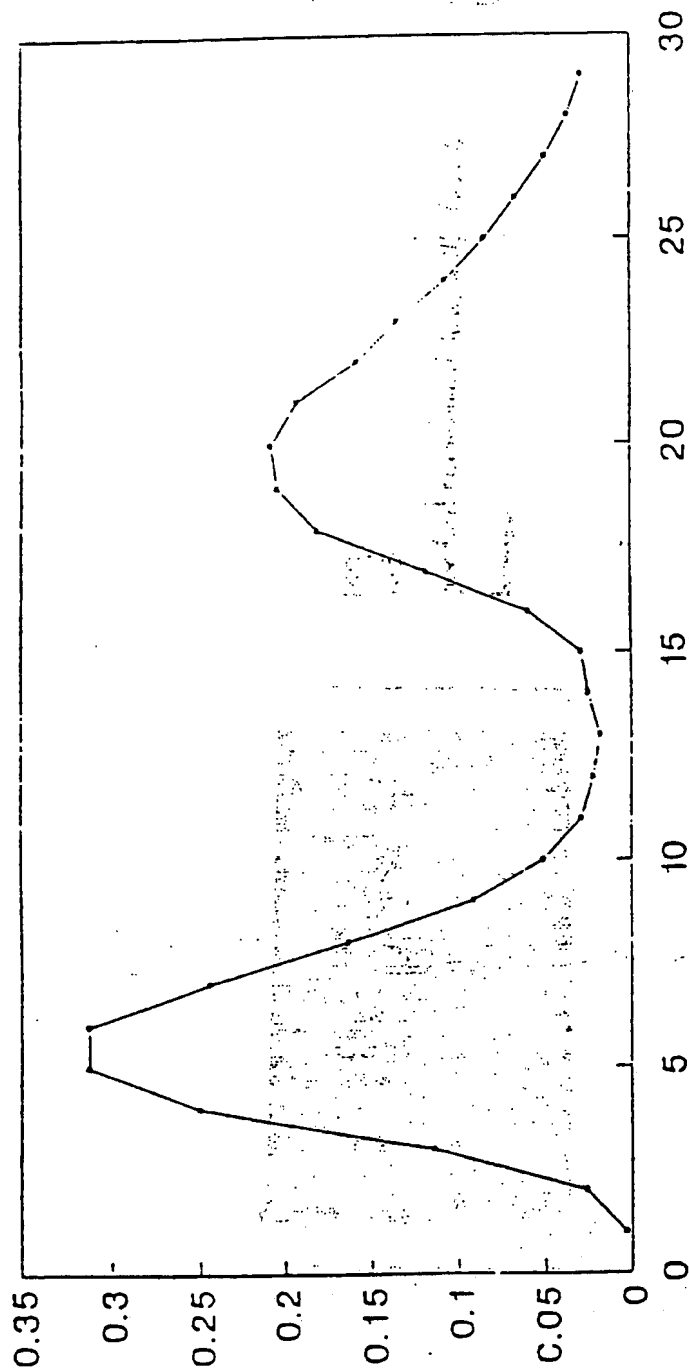
B





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50:50 Pig/Human Mix



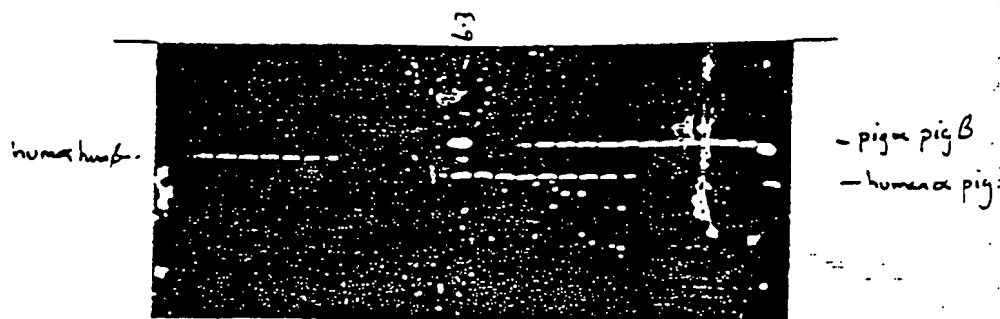
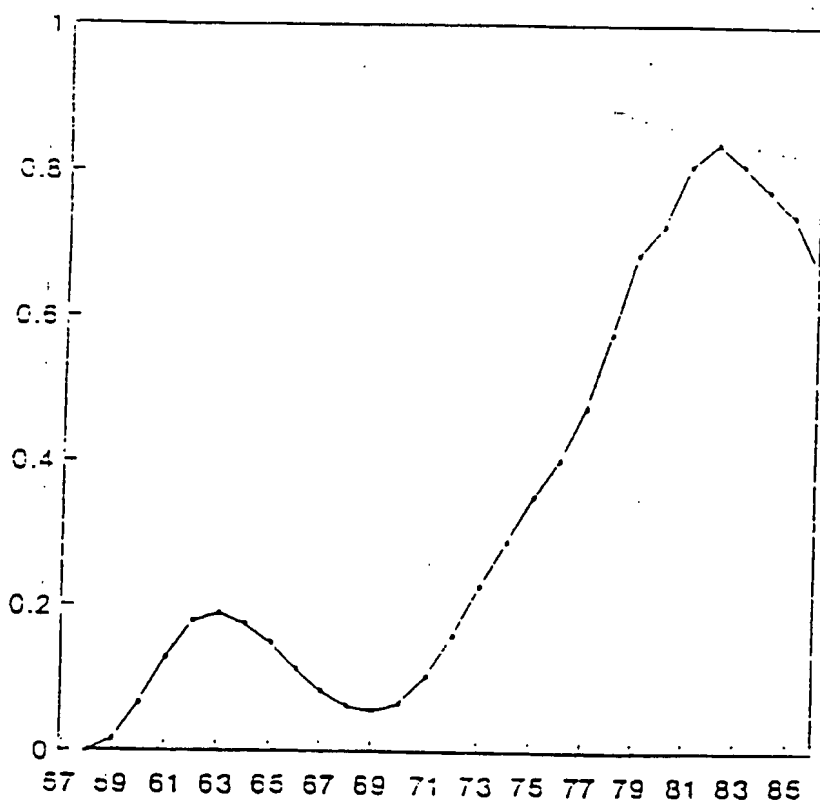
1:10 dilutions (129-2)

FIG. 4A

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FIG. 4B

Pig 6-3  
5 to 30 mM NaCl grad.



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# 50% Human - 50% Mouse Mix 5 to 30 mM NaCl grad.

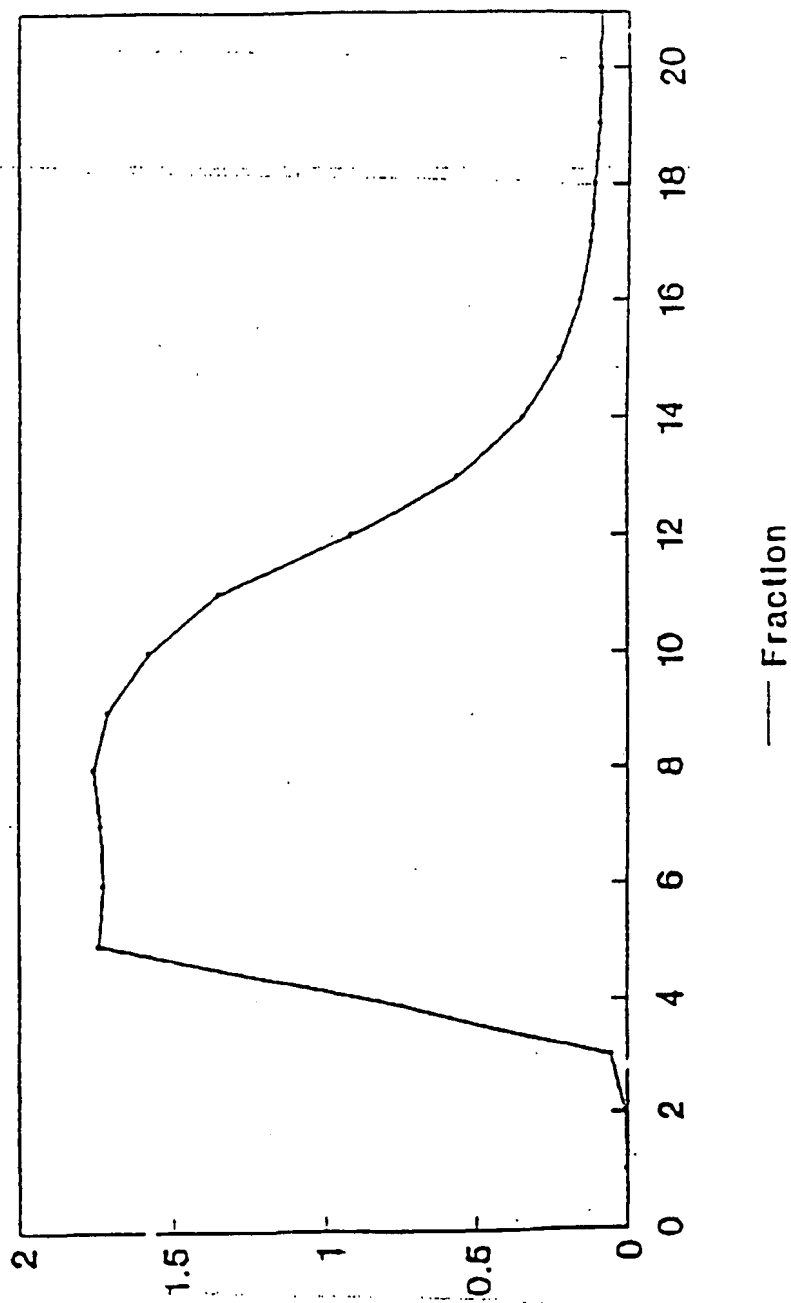


FIG. 4C

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FIG. 4D

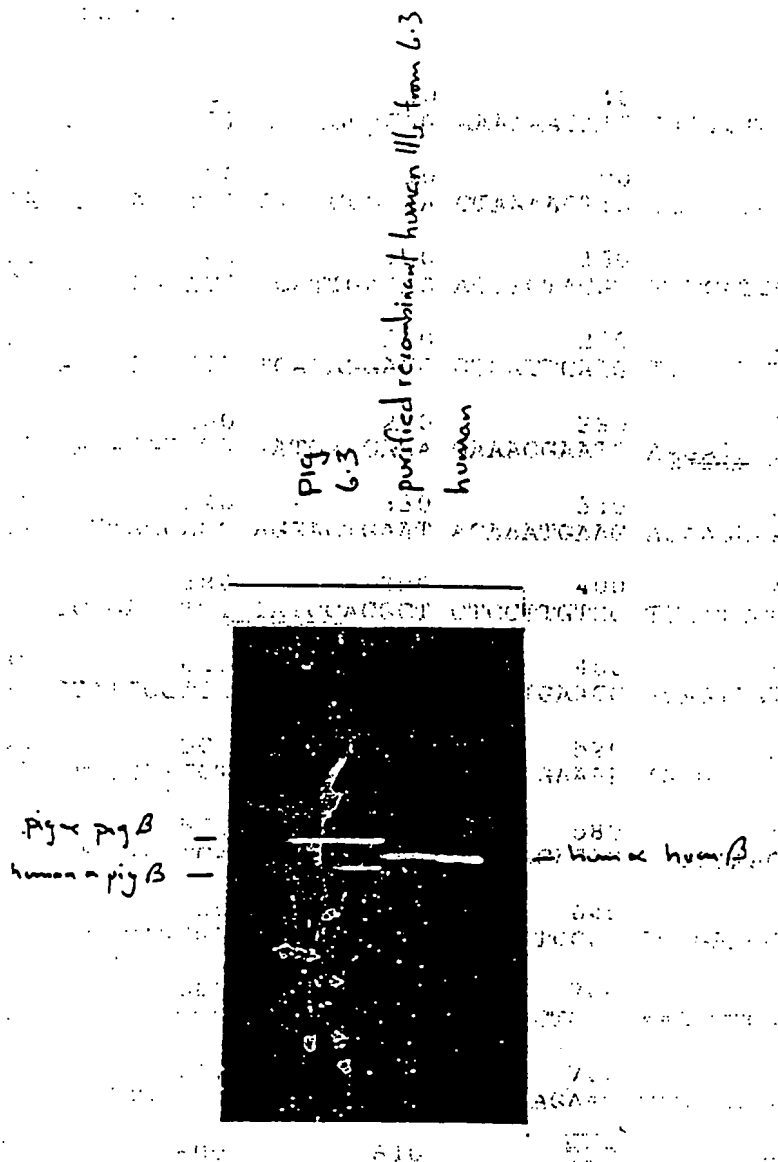
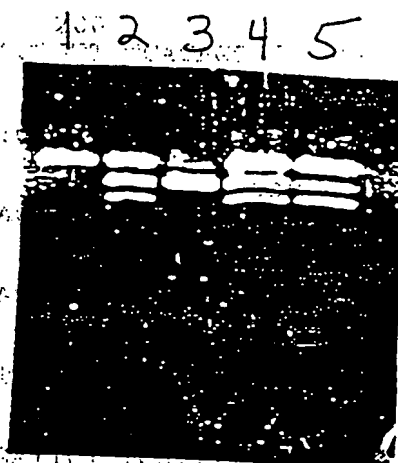
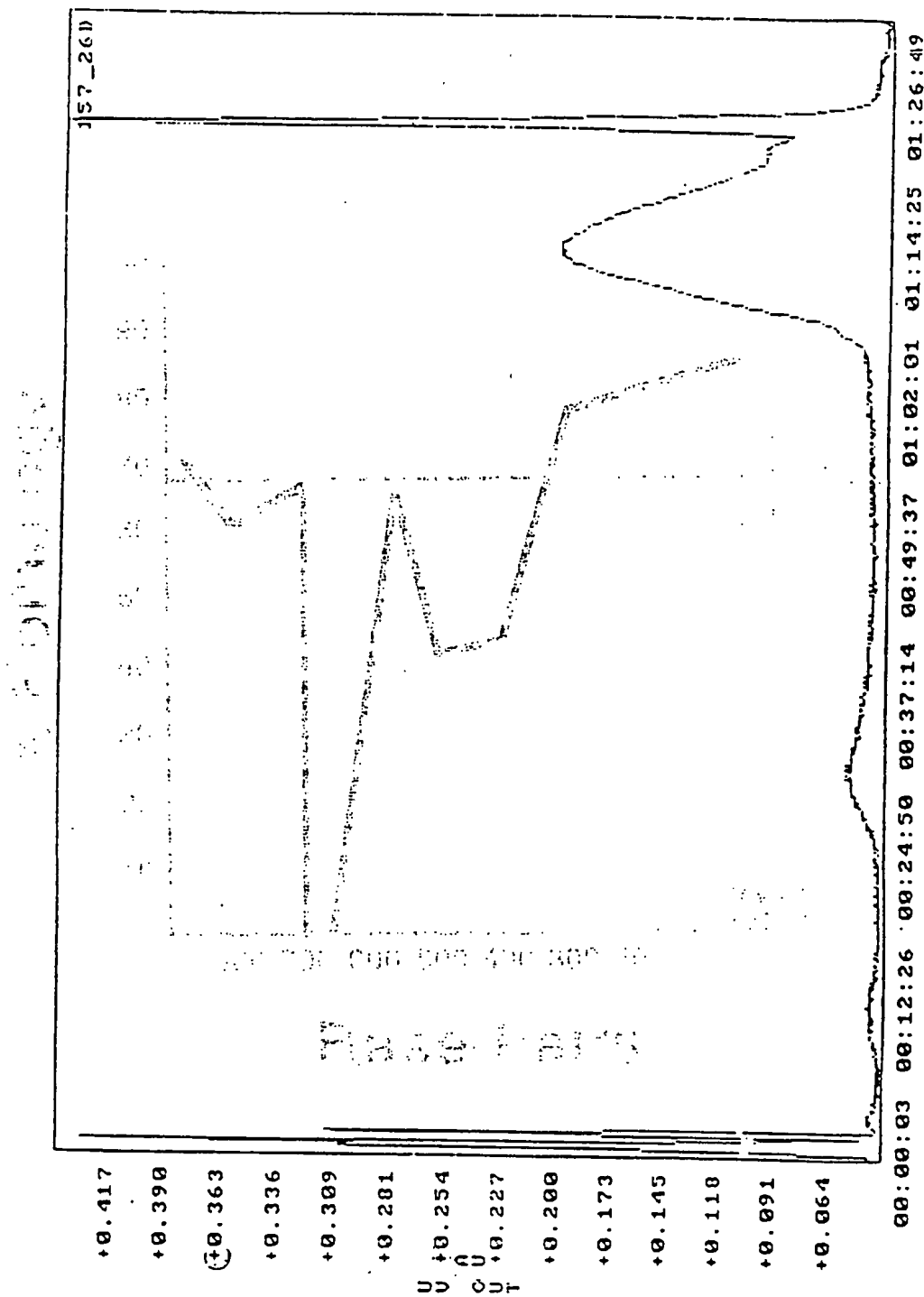


FIG. 5





TIME - hh:mm:ss

FIG. 6

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# Oxygen Affinity of Transgenic Hemoglobin

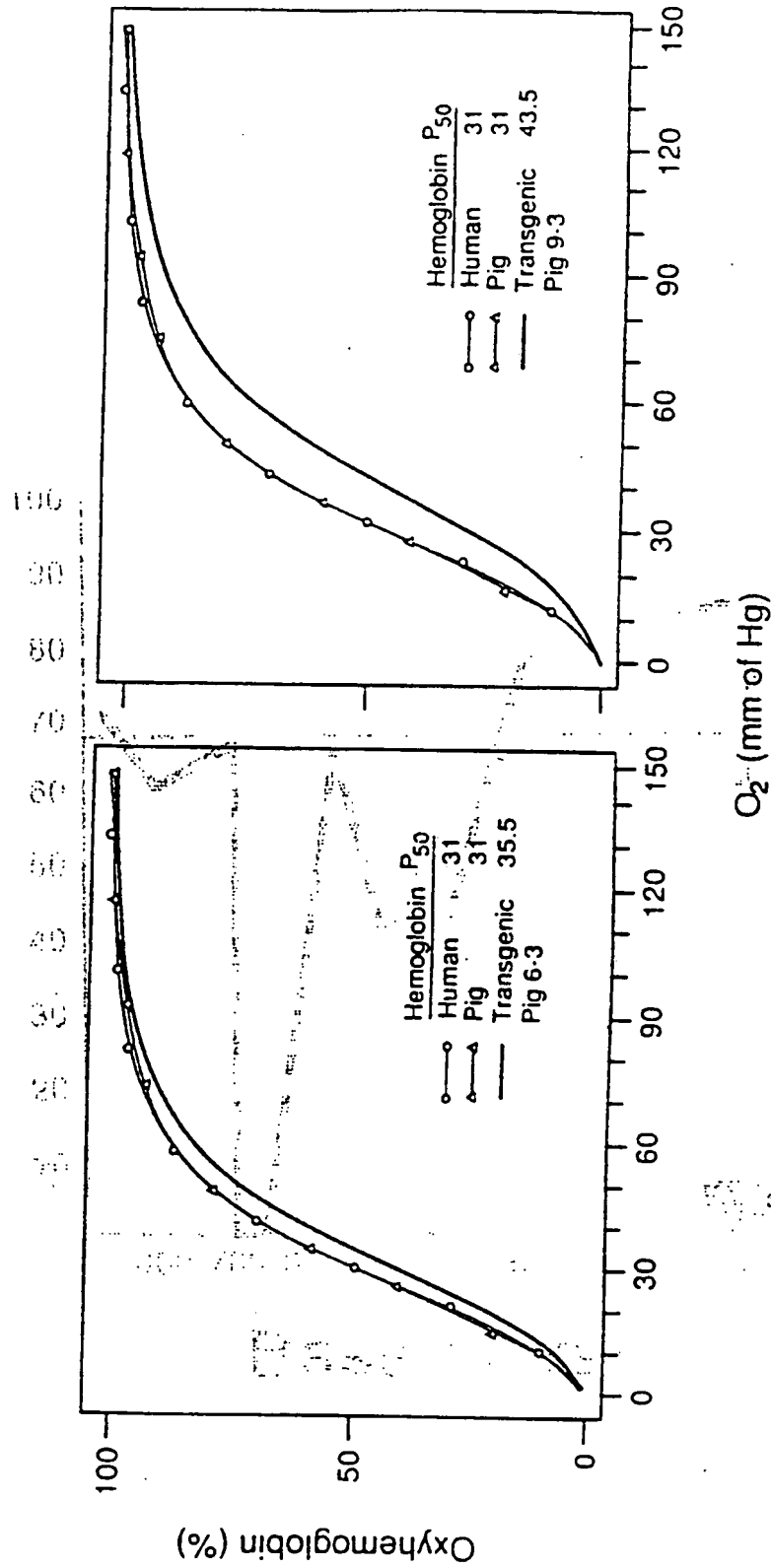


FIG. 7

## Adult pig globin promoter

10 20 30 40 50 60  
CCCCAGCCCT TTTTCCAGGT CAGCGCAGGG AAAAAACATG TTCTCTGTCC CTGGTTATAC  
70 80 90 100 110 120  
TGTTTAGAAA CATCACCTCC CTCGGCGAAA CTAAACTTG GGGGTTGCAA TTTATTCCTT  
130 140 150 160 170 180  
GCTTCTTTGT ATTTTCGTACC ACATTGAGAG AGCTCTAGGT TTTCATCCGC AGATTCCCAA  
190 200 210 220 230 240  
ACCTTCGCAG AGGAGCTGTT TCACAGGACC GTGATTCAAG TTTACTCTAC TTTTCCATCA  
250 260 270 280 290 300  
TTTATTTGGT CATATGTTTA AATGAAGAAA GAAAGGAATG AAGATACCTG AATGAAATGA  
310 320 330 340 350 360  
GTATTTGTTT TCTTACCAGC AGGACTGAAT ACAAATGAAG AGAAGAAAAA TACGCACATT  
370 380 390 400 410 420  
TAGGACTTGG GCAGAGGTTT TATCCACGCT CTCCTTGTGG TTATTTCCCA TATTCAGAAG  
430 440 450 460 470 480  
GCGCGGGTGT GGATTCGTCT GTATGGTCCT AAATTGAACC ACAGTGGTCA AATCCCTCCA  
490 500 510 520 530 540  
CTTTCTGCTC CTTGGATTCT TCGTTTGTGT ACTAAGAAAA TGGGGAGGCA GTCTCTAAGA  
550 560 570 580 590 600  
GATTGCTACA GTGGGACTCA ACTCTAAAAG TTGTACAGAC TTGCTAAGGA GGATGAAATT  
610 620 630 640 650 660  
AGTAGCACTT TGCACTGTGA GGATGGACCT AGAGCTCCCC AGAGAAGGGC TGAAGGTCTG  
670 680 690 700 710 720  
AAGTTGGTGC CAGGAACGTC TCGAAGACAG GTATACTGTC AACATTCAAG CCTCACCCTG  
730 740 750 760 770 780  
TGGAACCACG CCCTGGCCTG GGCCAATCTG CTCCCAGAAG CAGGGAGGGC AGGAGGCTGG  
790 800 810 820 830 840  
GGGGGCATAA AAGGAAGAGC AGAGCCAGCA GCCACCTACA TTTGCTTCTG ACACAACCGT  
850 860 870 880  
GTTCACTAGC AACTGCACAA ACAGACAACA TGGTGCATCT GTCTGCTGA

Figure 8



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1287 CCCCAGCCCTTTTCCAGGTCAGCGCAGGGAAAAACATGTTCTGTCCCTGGTTATAC  
 CCCCAGACACTCTTGCAGATTAGTCCAGGCAGAAA CA GTTAGATGTCCCCAGTTAACCC  
 61 TG T TTAGAAACATCACCTC CCTCGGCGAACTAAACCTTGGGGGTTGCAATTTATTC  
 1345 TCCTATTTGACACCACTGATTACCCCATTTGATAGTCACACTTTGGG TTGTAAGTGACTT  
 118 CTTGCTTCTTTGTATTTTCGTACCACATTGAGAGAGCTCTAGGTTTTTCATCCGCAGATTCC  
 1404 TTTATTTATTTGTATTTTGTACTGCATTAAGAGGTCTCTAGTTTTTTATCTCTTCTTCC  
 178 CAAACCTTCGCAGAGGAGCTGTTTCACAG G ACCGTGATTCAAGTTTACTCTACTTTTC  
 1464 CAAAACCTAATA AGTAACTAATGCACAGAGCACATTTCATTTGTATTATCTATTTTAA  
 236 GATCATTATTTGGTCATATCTTTAAATGAAGAAA 270  
 1523 GACATAATTTATTAGCATGCATGAGCAATTAAGAAA 1559  
 \* \* \* \* \*  
 Matches = 176 Length = 277 Matches/length = 63.5 percent  
 302 TATTGTTTTCTTACCAGCAGGACTGAATACAAATGAAGAGAAGAAAAA TACGCAC A  
 1629 TTTTCTTTTCTTACCAGAAGGTTTTAATCCAAATAAGGAGAAGATATGCTTAGAACTGA  
 359 TTTAGGACTTGGGCAGAGGTTTTATCCACGCTCTCCTTGTGGTTATTTCCCATATTCAGA  
 1689 GGTAG AGTTTT CATCCATTCTGTCTGTAGTATTT TGCATATCTGGAGACGCAGG  
 419 AGGCGCGGG TGTGGAT TCGT CTGTATGGTCTAAATTGAAC CACAGTGGTCAA  
 1746 AAGAGATCCATCTACATATCCCAAAGCTGAATTATGCTAGACAAAGCTCTTCCACTTTTA  
 472 ATCCCTCCACTTTCTGCTCCTTGGATTCTTCGTTTGTGTACTAAGAAAATGGGGAGGCAG  
 1806 GTGCATCAA TTTCTTATTTGTGTAATAAGAAAATGGGCAAAACGATCTTCAATATGCTT  
 532 TCTCTAA GAGATTGCTAC AGTGGG ACTCA ACTCTAAAATTGTACAGACTTGCTAA  
 1865 ACCAAGCTGTGATTCCAAATATTACGTAATACACTTGCAAAGGAGGATGTTTTAGTA  
 588 GGAGGATGAAATTAGTAGCACTTTGCACGTGTGAGG ATGG ACCTAGAGCTCCCCAGAGA  
 1924 GCAATTTGTACTGA TGGTATGGGGCCAAAGAGATATATCTTAGAGGGAGGGCTGAGGGTT  
 646 AGGGCTGAAGGTCTGAAGTTGGTGGCAGGAACGTCTCGAAGACAGGTATA CTGTCAACA  
 1983 TGAAGTCCAACCTCTAAGCCAGTGCCAGAAGAG C CAAGGACAGGTACGGCTGTCTATCA  
 705 TTCAAGCCTCACCTGTGGAACACGCGCTGGCCTGGGCCAATCTGCTCCAGAAAGCAGG  
 2041 CTTAGACCTCACCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCAGGAGCAGG  
 765 GAGGGCAGGAGGCTGGGG GGGCATAAAAGGAAGAGCAGAGCCAGCAGCCACCTACATT  
 2101 GAGGGCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATT  
 824 GCTTCTGACACAACCGTGTTCCTAGCAACTGCACAAACAGACAACATGGTGCATCTGTC  
 2161 GCTTCTGACACAACCTGTGTTCCTAGCAAC CTCAAACAGACACCATGGTGCACCTGAC  
 884 TGCTGA 889  
 2219 TCCTGA 2224

Figure 9.

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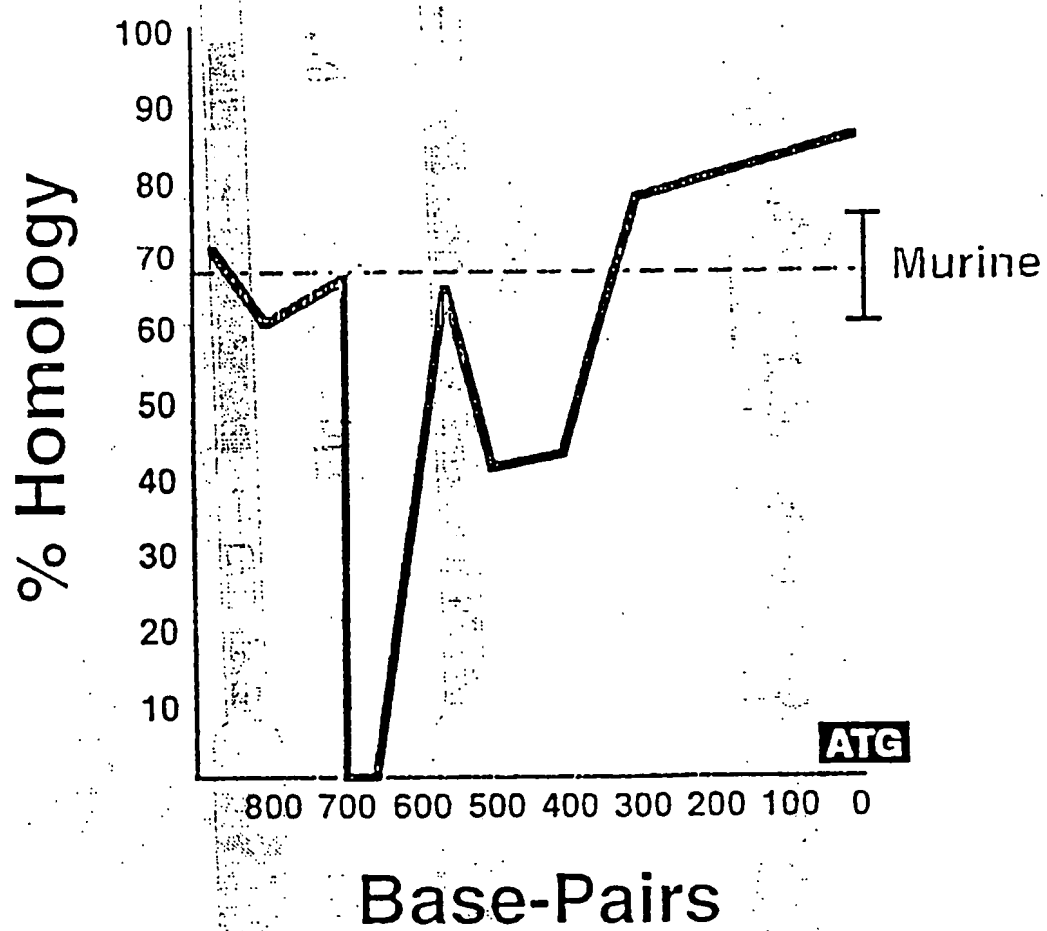


Figure 10.

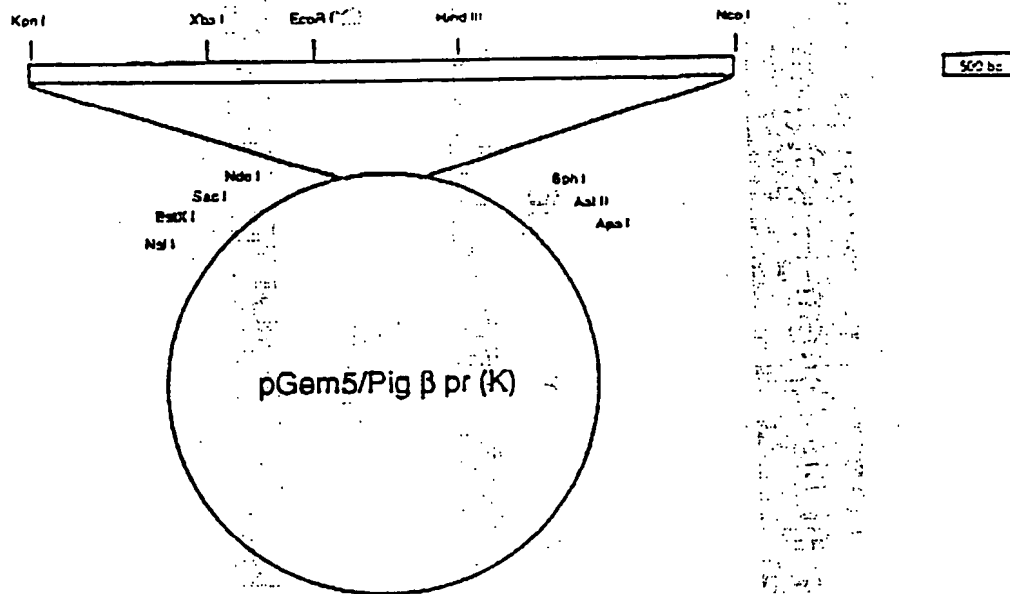


Figure 11.

Figure 12



$\beta^*$  = Pig promoter – Human  $\beta$  gene – Human 3' end  
 $\beta^{**}$  = Pig promoter – Human  $\beta$  gene – Pig 3' end

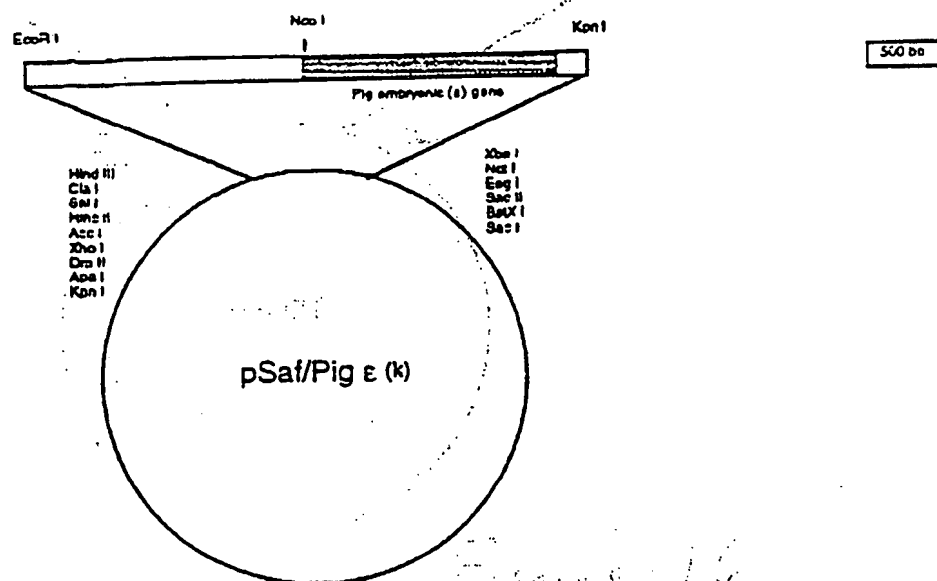


Figure 13.

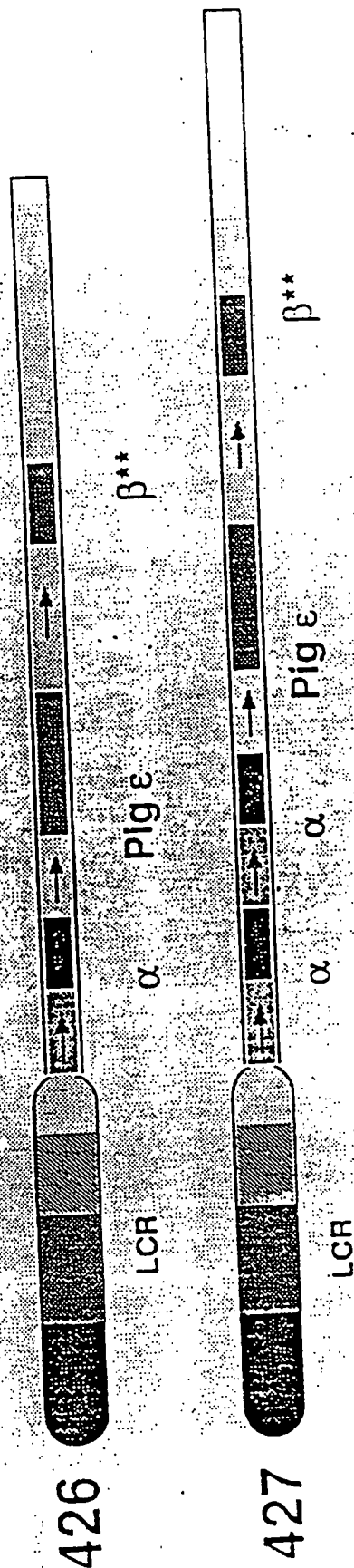


Figure 14.

$\beta^{**}$  = Pig promoter - Human  $\beta$  gene - Pig 3' end

# High Level Expression of Hemoglobin (Transgenic Pig)

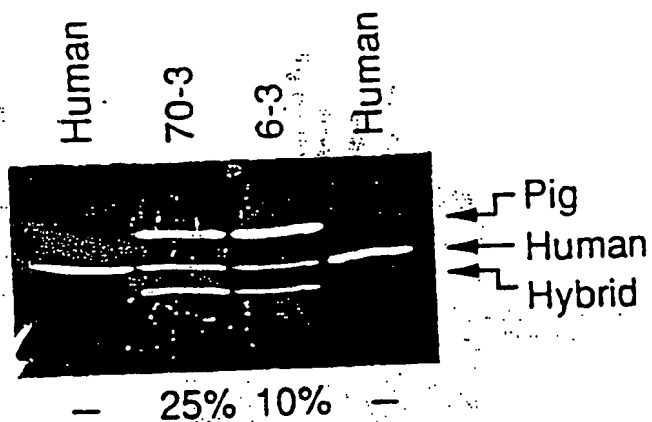


Figure 15

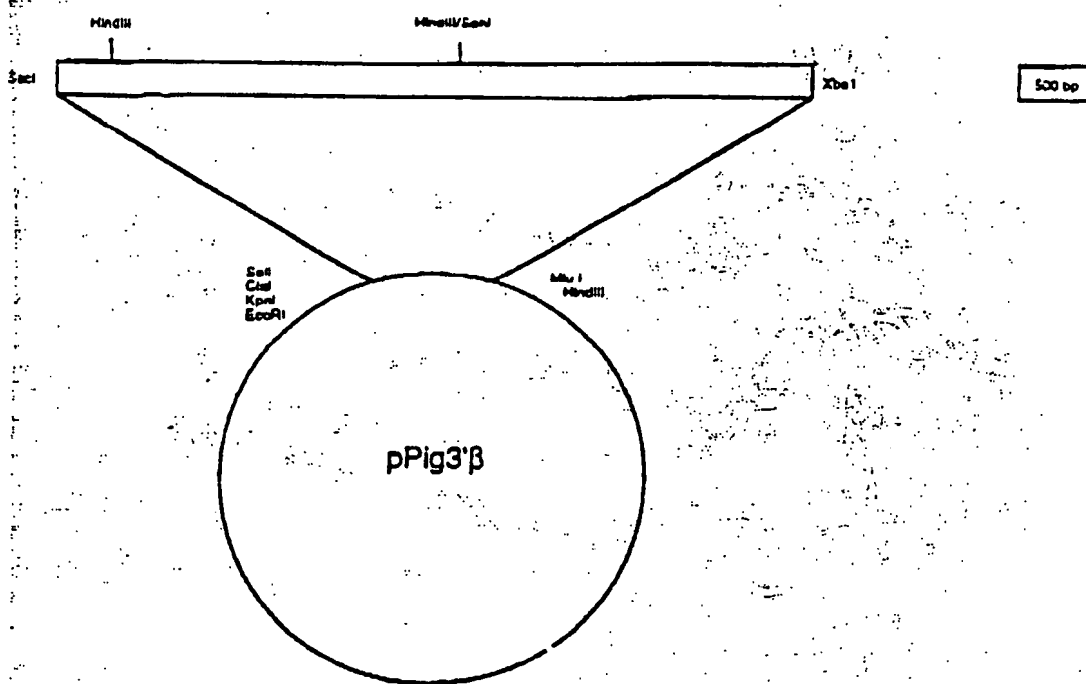


Figure 16.



## FIGURE 17

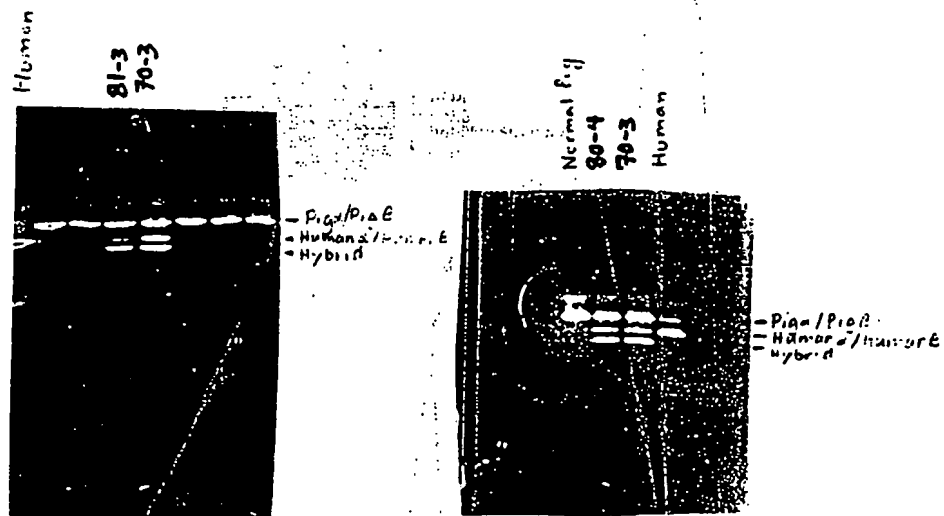
Transgenic pigs obtained from construct 339

Animal (Sex)	% Authentic Human Hb Expression	Copy #
70-3 (F)	23	3
80-4 (F)	18	3-4
81-3 (F)	5	n.d.

Hb: Hemoglobin

n.d: not determined

FIGURE 18



# FIGURE 19

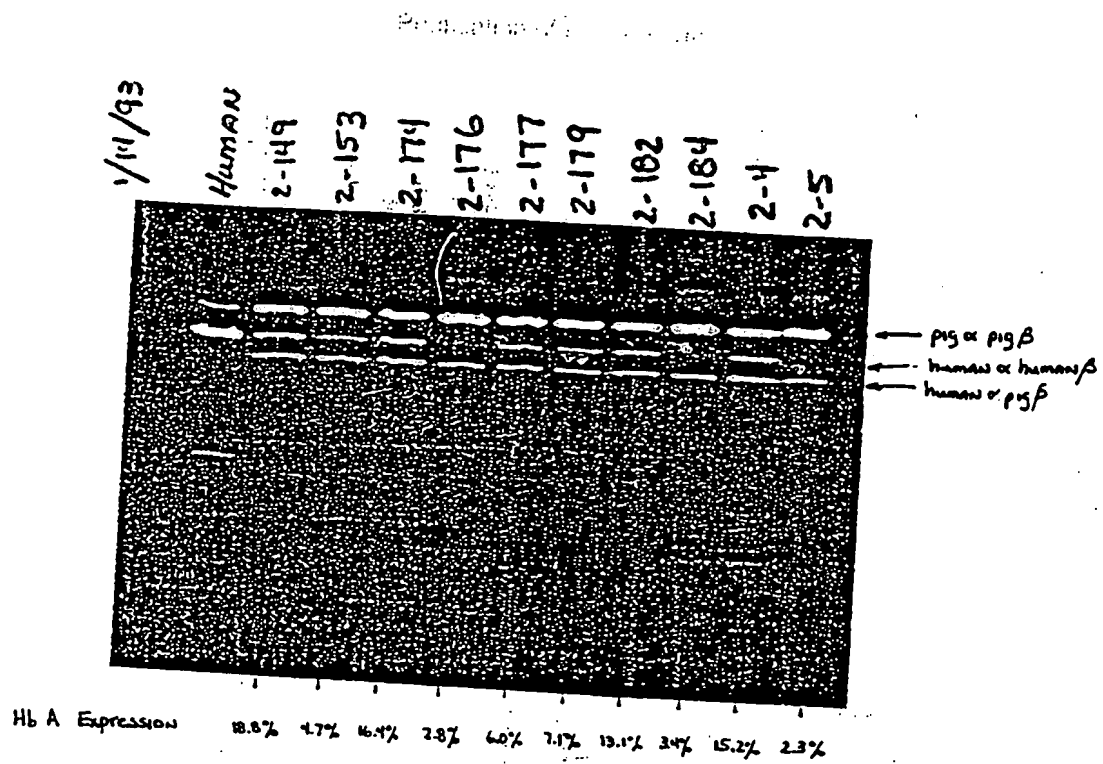


FIGURE 20

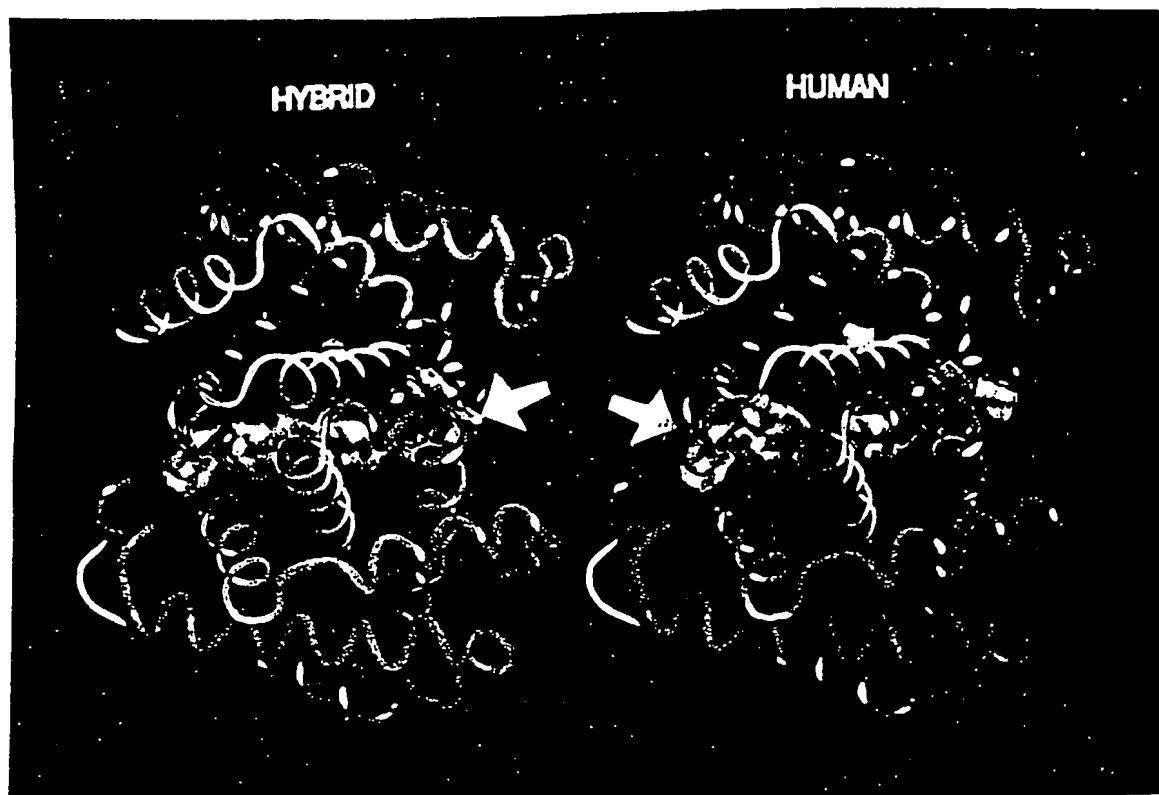
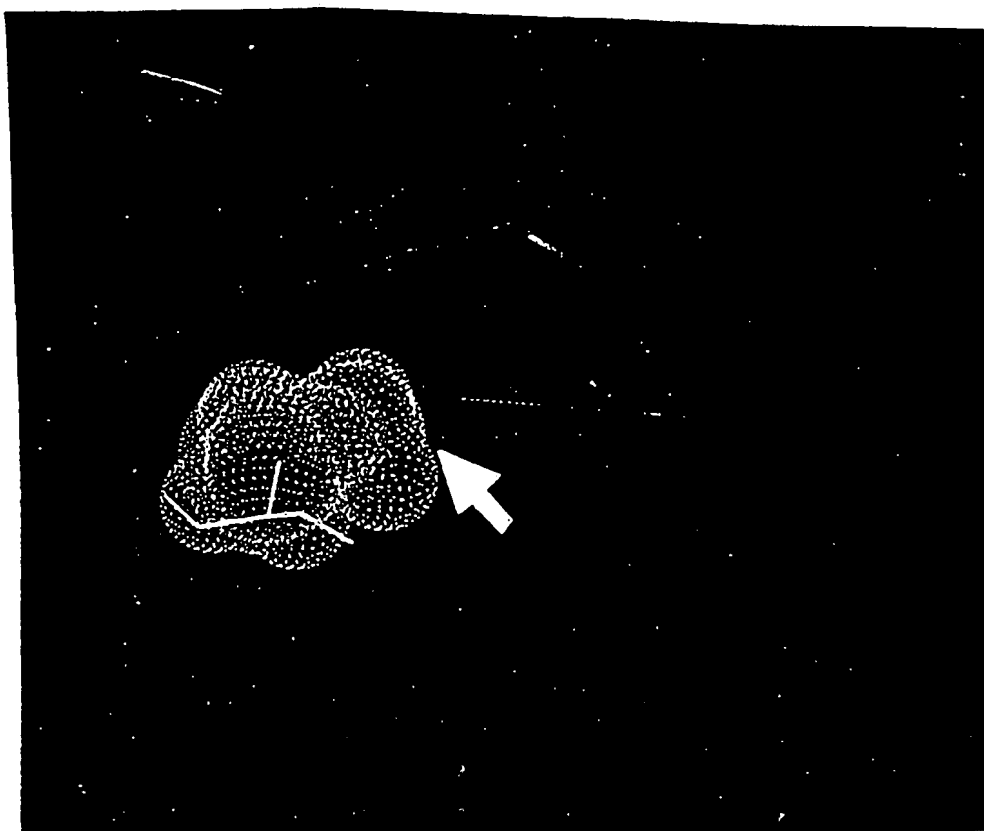


FIGURE 21

FIGURE 21



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2001 4575.1 1 1 31672 21.5, 24.1, 24.2 5371

forwarding to the U.S. Coast Guard (USCG) or to both national authorities, i.e., the

... 2000 年 12 月 31 日

1. *Formulation*—The first step in the identification process followed by classification is the

FIGURE 22

1. The first part of the document is a letter from the author to the reader, explaining the purpose of the document and the scope of the study. The letter is dated 1964 and is addressed to the reader.

1. The first group of countries is the "Group of 77" (G77), which consists of 119 developing countries. These countries are represented by the G77+China, which is a permanent member of the UNCTAD Governing Council.

1. *Corollary 1.1* Let  $\mathcal{A}$  be a  $\mathcal{C}^*$ -algebra and let  $\mathcal{K}$  be a  $\mathcal{C}^*$ -ideal of  $\mathcal{A}$ . Then the following conditions are equivalent:

10-11-57 10:00 AM 10:00 AM 10:00 AM

### Purification of Hb Presbyterian

Environ Biol Fish (2008) 81:185–195 | <http://dx.doi.org/10.1007/s10641-008-9418-1>

"Production of" "George Rabbin, alias and name", "Theodore J.  
ann 68-092 see page document".

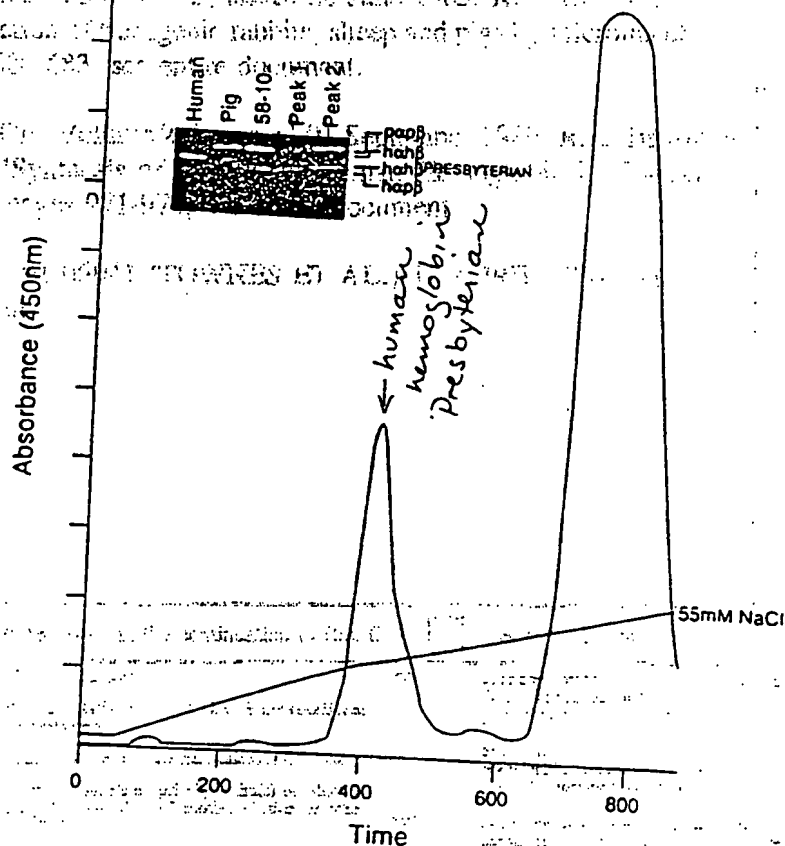
1 P 5 P P

... PRESBYTERIAN

0/147 [REDACTED] - napp  
Documents

100-101-102-103-104-105-106-107-108-109-110-111-112-113-114-115-116-117-118-119-120-121-122-123-124-125-126-127-128-129-130-131-132-133-134-135-136-137-138-139-140-141-142-143-144-145-146-147-148-149-150-151-152-153-154-155-156-157-158-159-160-161-162-163-164-165-166-167-168-169-170-171-172-173-174-175-176-177-178-179-180-181-182-183-184-185-186-187-188-189-190-191-192-193-194-195-196-197-198-199-200-201-202-203-204-205-206-207-208-209-210-211-212-213-214-215-216-217-218-219-220-221-222-223-224-225-226-227-228-229-230-231-232-233-234-235-236-237-238-239-240-241-242-243-244-245-246-247-248-249-250-251-252-253-254-255-256-257-258-259-260-261-262-263-264-265-266-267-268-269-270-271-272-273-274-275-276-277-278-279-280-281-282-283-284-285-286-287-288-289-290-291-292-293-294-295-296-297-298-299-300-301-302-303-304-305-306-307-308-309-310-311-312-313-314-315-316-317-318-319-320-321-322-323-324-325-326-327-328-329-330-331-332-333-334-335-336-337-338-339-340-341-342-343-344-345-346-347-348-349-350-351-352-353-354-355-356-357-358-359-360-361-362-363-364-365-366-367-368-369-370-371-372-373-374-375-376-377-378-379-380-381-382-383-384-385-386-387-388-389-390-391-392-393-394-395-396-397-398-399-400-401-402-403-404-405-406-407-408-409-410-411-412-413-414-415-416-417-418-419-420-421-422-423-424-425-426-427-428-429-430-431-432-433-434-435-436-437-438-439-440-441-442-443-444-445-446-447-448-449-450-451-452-453-454-455-456-457-458-459-460-461-462-463-464-465-466-467-468-469-470-471-472-473-474-475-476-477-478-479-480-481-482-483-484-485-486-487-488-489-490-491-492-493-494-495-496-497-498-499-500-501-502-503-504-505-506-507-508-509-510-511-512-513-514-515-516-517-518-519-520-521-522-523-524-525-526-527-528-529-530-531-532-533-534-535-536-537-538-539-540-541-542-543-544-545-546-547-548-549-550-551-552-553-554-555-556-557-558-559-560-561-562-563-564-565-566-567-568-569-570-571-572-573-574-575-576-577-578-579-580-581-582-583-584-585-586-587-588-589-590-591-592-593-594-595-596-597-598-599-600-601-602-603-604-605-606-607-608-609-610-611-612-613-614-615-616-617-618-619-620-621-622-623-624-625-626-627-628-629-630-631-632-633-634-635-636-637-638-639-640-641-642-643-644-645-646-647-648-649-650-651-652-653-654-655-656-657-658-659-660-661-662-663-664-665-666-667-668-669-670-671-672-673-674-675-676-677-678-679-680-681-682-683-684-685-686-687-688-689-690-691-692-693-694-695-696-697-698-699-700-701-702-703-704-705-706-707-708-709-710-711-712-713-714-715-716-717-718-719-720-721-722-723-724-725-726-727-728-729-730-731-732-733-734-735-736-737-738-739-740-741-742-743-744-745-746-747-748-749-750-751-752-753-754-755-756-757-758-759-760-761-762-763-764-765-766-767-768-769-770-771-772-773-774-775-776-777-778-779-780-781-782-783-784-785-786-787-788-789-790-791-792-793-794-795-796-797-798-799-800-801-802-803-804-805-806-807-808-809-810-811-812-813-814-815-816-817-818-819-820-821-822-823-824-825-826-827-828-829-830-831-832-833-834-835-836-837-838-839-840-841-842-843-844-845-846-847-848-849-850-851-852-853-854-855-856-857-858-859-860-861-862-863-864-865-866-867-868-869-870-871-872-873-874-875-876-877-878-879-880-881-882-883-884-885-886-887-888-889-890-891-892-893-894-895-896-897-898-899-900-901-902-903-904-905-906-907-908-909-910-911-912-913-914-915-916-917-918-919-920-921-922-923-924-925-926-927-928-929-930-931-932-933-934-935-936-937-938-939-940-941-942-943-944-945-946-947-948-949-950-951-952-953-954-955-956-957-958-959-960-961-962-963-964-965-966-967-968-969-970-971-972-973-974-975-976-977-978-979-980-981-982-983-984-985-986-987-988-989-990-991-992-993-994-995-996-997-998-999-1000-1001-1002-1003-1004-1005-1006-1007-1008-1009-1010-1011-1012-1013-1014-1015-1016-1017-1018-1019-1020-1021-1022-1023-1024-1025-1026-1027-1028-1029-1030-1031-1032-1033-1034-1035-1036-1037-1038-1039-1040-1041-1042-1043-1044-1045-1046-1047-1048-1049-1050-1051-1052-1053-1054-1055-1056-1057-1058-1059-1060-1061-1062-1063-1064-1065-1066-1067-1068-1069-1070-1071-1072-1073-1074-1075-1076-1077-1078-1079-1080-1081-1082-1083-1084-1085-1086-1087-1088-1089-1090-1091-1092-1093-1094-1095-1096-1097-1098

Quinn



CLASSIFICATION OF SUBJECT MATTER

50/52

INT. CL. 7: C07C 47/00; C12P 1/00; C12P 21/00  
 C12C 43/00; C12C 43/20; C12C 43/24; C12C 43/26

According to International Patent Classification (IPC) and to the International Patent Classification (IPC) for the purpose of the present invention.

B. FIELDS SEARCHED

Minimum documentation searched (classification):

INT. CL. 7: C07C 47/00; C12P 1/00; C12P 21/00; C12C 43/00; C12C 43/20; C12C 43/24; C12C 43/26

Documentation searched (classification):

FIGURE 23

Documentation searched (classification):

DIOSIE, AM, CA

Documentation searched (classification):

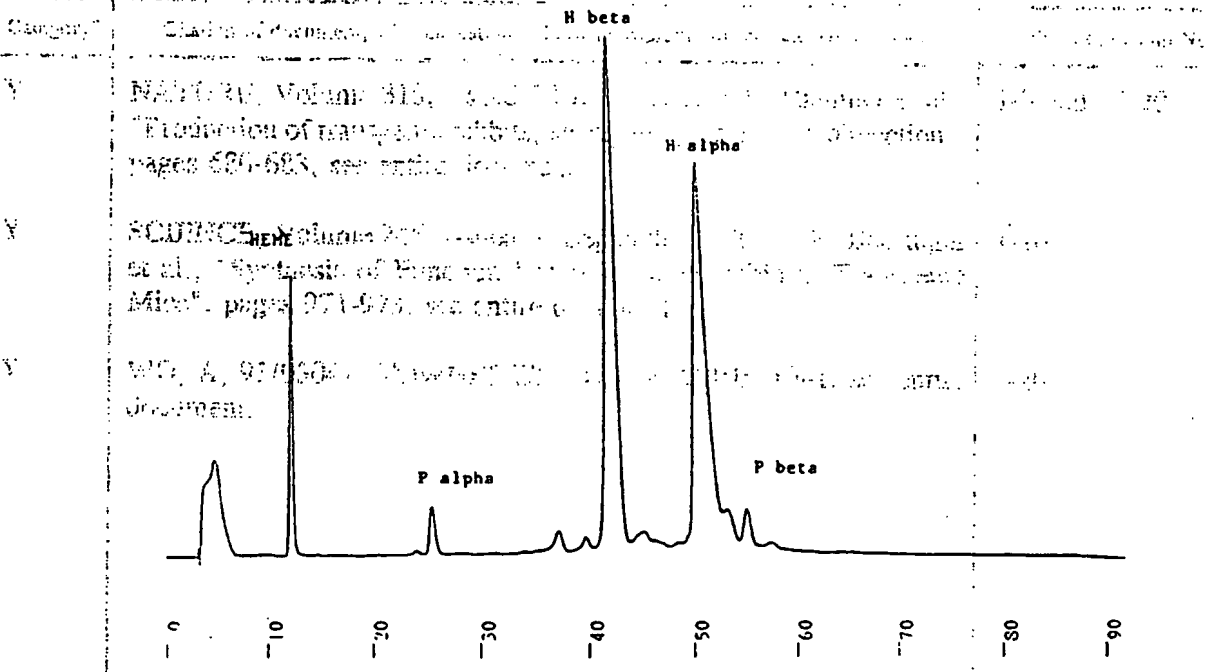
C. REFERENCES CITED TO BE TAKEN INTO ACCOUNT

Category: Chemical compounds, processes, and products

Y NATURE, Volume 315, 1980, 1-10. "Production of many... pages 686-688, see article for details.

Y SOURCE: "Synthesis of... pages 971-973, see article for details.

Y WOL, A, 91/1504, 1991, 1-10. "Production of many... pages 686-688, see article for details.



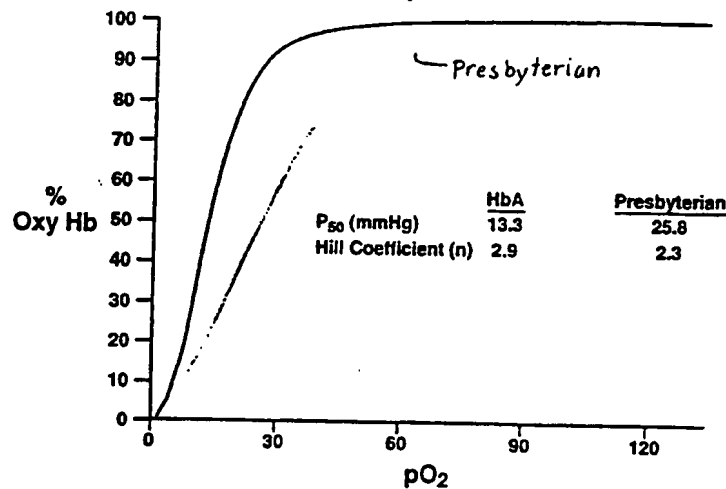
1. The present invention relates to the synthesis of...  
 2. The present invention relates to the synthesis of...  
 3. The present invention relates to the synthesis of...  
 4. The present invention relates to the synthesis of...  
 5. The present invention relates to the synthesis of...  
 6. The present invention relates to the synthesis of...  
 7. The present invention relates to the synthesis of...  
 8. The present invention relates to the synthesis of...  
 9. The present invention relates to the synthesis of...  
 10. The present invention relates to the synthesis of...

Continued on DOCUMENTS CONSIDERED TO BE REL 51/52

Category: Condition of treatment, with indication where appropriate of the type of treatment.

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, volume 79, number 22, pp. 6831-6834, 1982, 24 p. Translated in: "Biochemical aspects of aging" (ed. by J. H. Birrell, 1982, pp. 643-648, see earlier article).

### Oxygen Binding Curve Hb Presbyterian

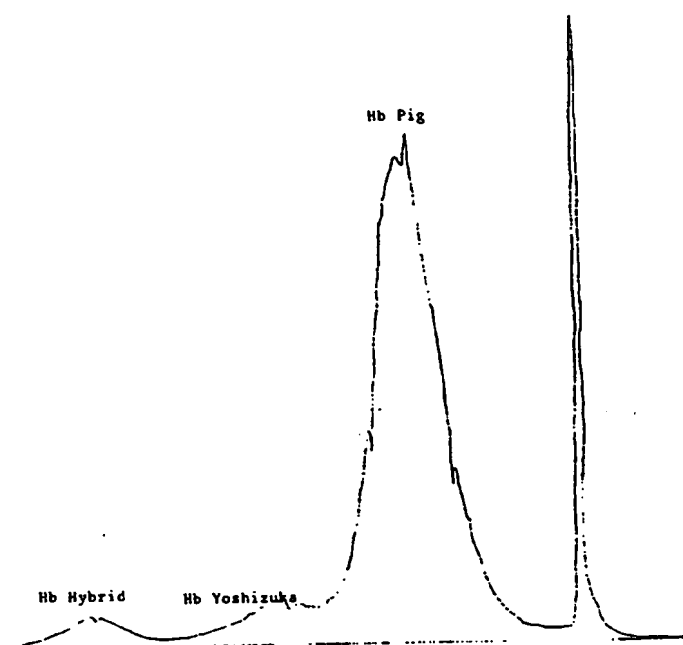




2 (Continuation) DOCUMENTS CONTAINED HEREIN ARE 52/52

Category	Classification of document	Relevance to the invention
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Y	PROCEEDINGS OF THE AMERICAN ACADEMY OF SCIENCE, Vol. 10, No. 1, 1991, pp. 1-10. D.I. Proulx, <b>FIGURE 25</b> , pp. 1-10.	11
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Best Available Copy  
INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US93/05629

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : A01K 67/00, 67/027; C12N 15/90; C12P 21/06  
US-CL : 435/69.1, 69.6; 536/23.1; 23.5, 24.1, 24.2; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.6; 536/23.1; 23.5, 24.1, 24.2; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, APS, CA

search terms: transgen?; pig?; porcine; hemoglobin; globin; epsilon; purif?; resin#; ion (w) exchange; Q; human; cdna; genom?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 315, issued 20 June 1985, R.E. Hammer et al., "Production of transgenic rabbits, sheep and pigs by microinjection", pages 680-683, see entire document.	1-3 and 13-20
Y	SCIENCE, Volume 245, issued 01 September 1989, R.R. Behringer et al., "Synthesis of Functional Human Hemoglobin in Transgenic Mice", pages 971-973, see entire document.	1-20
Y	WO, A, 91/05041 (TOWNES ET AL.) 18 APRIL 1981, see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be part of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* A	document member of the same patent family

Date of the actual completion of the international search

08 September 1993

Date of mailing of the international search report

20 SEP 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

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Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

WD 83/1567

International application No.

PCT/US93/05629

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, Volume 86, issued September 1989, T. Enver et al., "The human $\beta$ -globin locus activation region alters the developmental fate of a human fetal globin gene in transgenic mice", pages 7033-7037, see entire document.	1-20
Y	JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, Volume 14, issued 1987, C. Gelfi et al., "Purification of human hemoglobin valence intermediates by preparative immobilized pH gradients", pages 129-147, see entire article.	18 and 20
Y	JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, Volume 17, issued 1988, S.M. Christensen et al., "Preparation of human hemoglobin Ao for possible use as a blood substitute", pages 143-154, see entire article.	1-20
Y	JOURNAL OF CHROMATOGRAPHY, volume 487, issued 1989, F. Kutlar et al., "QUANTITATION OF HEMOGLOBIN BART'S, H, PORTLAND-I, PORTLAND-II AND CONSTANT STRING BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY", pages 265-274, see entire article.	18 and 20
Y	JOURNAL OF CHROMATOGRAPHY, volume 427, issued 1988, C.T.A. Evelo et al., "Separation of human haemoglobin alkylated at $\beta$ 93 cysteine from its native form by fast protein liquid chromatography", pages 335-340, see entire article.	18 and 20
Y	JOURNAL OF CHROMATOGRAPHY, volume 359, issued 1986, D.J. Burke et al., "RAPID CATION-EXCHANGE CHROMATOGRAPHY OF HEMOGLOBINS AND OTHER PROTEINS", pages 533-540, see entire article.	18 and 20
Y	E. ANTONINI et al. "METHODS IN ENZYMOLOGY, VOLUME 76, HEMOGLOBINS", published 1981 by ACADEMIC PRESS (N.Y.), see pages 97-125, see entire excerpt.	18 and 20
Y	CELL, volume 38, issued August 1984, S. Wright et al., "DNA Sequences Required for Regulated Expression of $\beta$ -Globin Genes in Murine Erythroleukemia Cells", pages 265-273, see entire article.	10-12

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p><b>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, volume 76, number 11, issued November 1979, N.J. Proudfoot et al., "Molecular cloning of human epsilon-globin gene", pages 5433-5439, see entire article.</b></p> <p>The present invention is directed to a transgenic pig for the production of recombinant human hemoglobin. The transgenic pig is used as an efficient and economical source of human hemoglobin and other proteins. The transgenic pig is produced by introducing a recombinant DNA molecule into a pig embryo. The recombinant DNA molecule contains a human epsilon-globin gene and a pig beta-globin gene. The transgenic pig is then bred to produce offspring that produce human hemoglobin.</p> <p>2. BACKGROUND OF THE INVENTION</p> <p>2.1. HEMOGLOBIN</p> <p>Oxygen absorber through the use of hemoglobin in red blood cells. Hemoglobin is a protein that is found throughout the body. It is responsible for the transport of oxygen from the lungs to the tissues. Each hemoglobin molecule consists of four polypeptide chains, two alpha chains and two beta chains. Each chain contains a heme group, which is a prosthetic group that contains an iron atom. The iron atom is coordinated to the oxygen molecule, which is transported by the hemoglobin molecule.</p> <p>The present invention is directed to a transgenic pig for the production of recombinant human hemoglobin. The transgenic pig is used as an efficient and economical source of human hemoglobin and other proteins. The transgenic pig is produced by introducing a recombinant DNA molecule into a pig embryo. The recombinant DNA molecule contains a human epsilon-globin gene and a pig beta-globin gene. The transgenic pig is then bred to produce offspring that produce human hemoglobin.</p>	11.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/05629

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
(Telephone Practice)

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

- I. Claims 1-3 and 13-20, drawn to transgenic pigs and methods of making hemoglobin, classified in Class 800, subclass 2 and Class 435, subclass 69.6.
- II. Claim 10, drawn to a  $\beta$ -globin promoter, classified in Class 536, subclass 24.1.
- III. Claim 11, drawn to a pig epsilon gene, classified in Class 536, subclass 23.5.
- IV. Claim 12, drawn to the 3'-non-coding region of the pig adult  $\beta$ -globin gene, classified in Class 24.1.

The inventions are distinct, one from the other for the following reasons:

The invention of group I is distinct from the inventions of Groups II-IV because they are drawn towards materially different compositions. For example, the compositions of group I comprise transgenic pigs whereas the compositions of the other three groups are drawn to nucleic acids. Further, the transgenic compositions are characterized in that they express human hemoglobin genes while the nucleic acid compositions of groups II-IV are derived from porcine genes.

The inventions of Groups II-IV are distinct one from the other because they are drawn to materially different elements of porcine nucleic acid. For example, the nucleic acid of group I comprises the promoter region for the porcine  $\beta$ -globin gene, whereas the nucleic acid of Group II comprises the structural gene for the porcine epsilon gene which is chemically unrelated to the  $\beta$ -globin locus. The invention of Group IV is directed towards a non-coding region of the porcine  $\beta$ -globin gene which does not mediate any physical process such as transcription and is therefore distinct from the promoter region of Group II.

In addition, the compositions of Groups II-IV may be used for materially different purposes other than the generation of transgenic animals, such as the production of recombinant proteins *in vitro*. Therefore, the four inventions listed above lack any special technical feature within the meaning of PCT Rule 13.2, linking them so as to constitute a unified invention.

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